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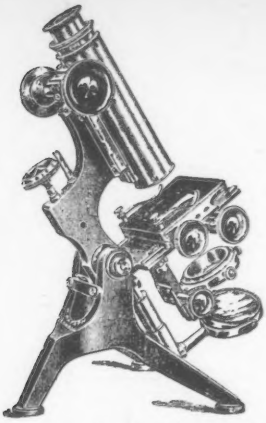

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XXV. BIOCHEMICAL CHANGES DUE TO ENVIRONMENT.

By OTTO ROSENHEIM.

From the Physiological Laboratory, King's College, London.

(*Received August 10th, 1918.*)

Observations on the influence of climate on the adaptation of alpine plants have so far been limited to the more or less obvious macroscopic or microscopic changes and their accompanying physiological effects on assimilation, transpiration and respiration. The most extensive investigations were those of Bonnier [1888, 1894] carried out in the experimental gardens in Paris and in the Montblanc range (1060–2800 m.), and those of Kerner [1894] who grew plants from seed in the botanical garden of Vienna and on the summit of the Blaser (2195 m.), near Innsbruck. The main conclusions to be drawn from this work were that the whole habit of the plants grown at higher altitudes became more stunted and dwarfed than in the plains, the development of the subterranean parts was relatively greater and the internal structure of both stem and leaf was modified, tending to lead to an increased power of assimilation. Of the principal factors of the alpine climate which gave rise to these changes the largest share is ascribed to the more intense illumination by day accompanied by low night temperatures and a drier atmosphere. Many observers as well as Kerner and Bonnier note the greater intensity of colour of alpine flowers and Bonnier attempted to confirm this subjective observation by comparing the colour tints of flowers at different altitudes in the mountains of Austria-Hungary by means of a graduated colour scale or "chromometer."

The rapid progress which has been made in the last few years in our knowledge of the chemistry of flower pigments makes it possible to attack these questions from a different point of view. It was first suggested by Miss Wheldale [1909] that a relationship exists between the anthocyanins and the flavones (or possibly xanthones). Whilst the theoretical assumptions of Miss Wheldale [1911] demanded hydrolytic and oxidative processes for

the formation of anthocyanins from flavone glucosides, the experimental facts subsequently brought forward by Keeble, Armstrong and Jones [1913] led them to assume an interplay of reducing and oxidative enzymes. Combes [1913] was the first completely to contradict the oxidation theory and he showed that a reducing agent, presumably an enzyme, plays the fundamental rôle in the process. Everest [1914] confirmed this conclusion and was able to convert extracts of white and yellow flowers, probably containing flavone glucosides, without intermediate hydrolysis, into anthocyanins by making use of reducing agents, of which magnesium was the most suitable. Willstätter, in conjunction with Everest [1913] and with Mallison [1914] adduced definite proofs that all naturally occurring anthocyanins were glucosides of reduced flavones or flavonols, and furnished on hydrolysis the chromophoric component (anthocyanidin = reduced flavone or flavonol) and a carbohydrate (glucose or galactose)[1].

Shibata [1915] pointed out the universal distribution of flavone derivatives in the overground parts of plants, and in conjunction with Nagai and Kishida [1916] showed that alpine and tropical plants especially are rich in these substances. The Japanese investigators advanced the interesting suggestion that the presence in the peripheral tissue of flavone derivatives may possess an important physiological significance in absorbing the ultra-violet rays of sunlight and thus protecting the plant against the injurious action of these rays. They also demonstrated the fact that greatly diluted solutions of chemically pure flavone derivatives absorb the ultra-violet region of the iron arc light almost completely.

If the hypothesis of Shibata is correct one would expect that alpine plants grown at lower altitudes, in which ultra-violet light is known to be much less intense owing to its rapid absorption by the atmosphere, would not produce the flavone substances to the same extent as those grown in the Alps where the insolation is so much more intense.

In spite of the apparently adverse conditions (dampness of the atmosphere in winter and the absence of the protecting influence of a permanent layer of snow) alpine plants can be easily grown in this country and develop normally from the seedling to the flowering and fruiting stage, even in the neighbourhood of London where they are exposed to the influence of a smoke-laden atmosphere. One of the easiest to grow from seed is the well known Edelweiss (*Leontopodium alpinum*) and partly for this reason and partly because I had

[1] For detailed discussion and full literature of this subject see the monographs of Everest [1915] and Wheldale [1916].

a considerable number of specimens collected by myself in the Alps available for comparison, I selected this plant for the experiments to be described.

A large number of flowers from plants growing on my rock garden in a London suburb (80 m. above sea) were used in these experiments. They came from a plant collected six years ago on Mt. Colbricon, about 2000 m. above sea level (near San Martino di Castrozza), and from the seedlings of this plant, of which several generations had been raised. The plants are growing in different positions as regards aspect and soil, but in all cases care was taken that the soil contained a plentiful supply of calcium carbonate (mortar rubble), which is essential for the well-being of this and the majority of alpine plants.

The flowers which served for comparison with the lowland flowers were collected during a period extending over several years from various places in the central and eastern Alps (Switzerland, Tyrol, Dolomites), generally at a height of 2000 m., and had been preserved in the usual way[1].

The whole of the material was gathered approximately at the same stage of development of the plants (during middle and end of August) and was finally dried together for one month *in vacuo* over sulphuric acid.

The presence of flavone derivatives in the silvery hairs of the bracts of the Edelweiss inflorescence can easily be demonstrated by two reactions. An intense yellow coloration is at once produced on exposure of the flowers to ammonia vapour, a characteristic reaction of nearly all white flowers which was first made use of by Miss Wheldale. The second reaction is still more striking and may be demonstrated with as little as 0·2 g. of the flower bracts. The colourless, or slightly yellowish, alcoholic extract of the bracts gives on reduction with magnesium in acid solution (in the presence of mercury as a catalyst) an intense orange-red colour [Shibata, 1915].

Nature of the Chromogenic Substance. The isolation and identification of the chromogenic substance obviously demands a larger amount of material than was at my disposal. There can be little doubt that the substance belongs to the class of flavone derivatives, which occur in plants in the free state as well as in glucosidic combination. A very simple reaction makes it possible to decide whether the glucoside, the non-glucoside, or a mixture of the two is present in Edelweiss. In their work on flower pigments Willstätter and Everest [1913] devised the so-called "anthocyanidin" reaction which depends

[1] It had already been noticed by Shibata [1915] that flavones are not lost appreciably if the drying is done quickly, and that determinations can be made with any well preserved herbarium material, even if kept for a number of years.

on the fact that the glucoside (anthocyanin) remains entirely in dilute sulphuric acid solution when shaken with amyl alcohol, whilst the non-glucoside (anthocyanidin) passes quantitatively into the amyl alcohol layer. When this alcoholic layer is shaken with sodium carbonate solution the pigment descends quantitatively into the aqueous layer. It is apparent that this reaction may be applied to the reduced coloured extracts, in which the flavone or its glucoside originally present, is reduced either to anthocyanidin or anthocyanin. It was found that plants collected in the Alps contained traces of the chromogenic substance in glucosidic combination, whilst the London grown plants contained only free flavones[1]. The reductions were carried out at a low temperature in order to avoid any acid hydrolysis of the glucoside, and the reduced solutions were diluted with water and freed from alcohol by shaking out with ether. On shaking with amyl alcohol the pigment passed quantitatively into it in the case of the homegrown plants, whilst a faint tint remained in the other cases even after repeated extraction with fresh amyl alcohol. The alcoholic solution showed the typical spectral band between λ 480–λ 520, allowing red and violet to pass. On shaking the amyl alcohol solution with sodium acetate the pigment remained in the alcohol, but it descended quantitatively into the aqueous layer on shaking with dilute sodium carbonate. The conclusion that the chromogen is present as a free flavone and not in glucosidic combination receives a certain amount of confirmation from the solubilities of the substance (insoluble in water, soluble in alcohol).

Quantitative Relationship of Flavone Derivatives in Edelweiss grown in London and in the Alps. The colour produced by the reduction of the colourless alcoholic extract of Edelweiss obviously offers a method for comparing quantitatively the amounts of flavone derivatives formed in the plant under different environmental conditions. This may be carried out either by comparing the colour intensity of the various extracts *inter se* or with a standard solution. The finding of a suitable standard makes the latter procedure difficult. An attempt was made to use the colour produced on reducing alcoholic apigenin (1. 3. 4′-trihydroxyflavone)[2] solutions of known strength by means of magnesium. The colour of the reduced solution, although

[1] The white flowers of alpine saxifrages and of *Dryas octopetala* also contained free flavones only. The majority of white non-alpine flowers which I examined, on the other hand, contained practically the whole of their chromogen in the glucosidic combination.

[2] Apigenin was obtained by the hydrolysis of the glucoside apiin, which I prepared from parsley. I am indebted to Dr Barger for another sample of pure apiin, which gave identical results.

resembling that of the reduced Edelweiss extracts to the naked eye, was found on comparison in Duboscq's colorimeter to differ in its tint to such an extent as to make exact comparison impossible.

The complete extraction of the flavone at first offered some difficulties. Exhaustive extraction with alcohol in a Soxhlet's apparatus introduced variable amounts of greenish-yellow decomposition products of chlorophyll which could not be completely removed by shaking out with light petroleum. Although small in amount they were sufficient to influence the tint of the reduction colour. The following procedure was finally adopted. Quantities of 0·5 or 1·0 g. of the plant organs were repeatedly extracted with twenty parts of 90 per cent. alcohol at 70–75° for some minutes, until the decanted extract no longer gave any colour after reduction. At least six extractions were found to be necessary. Each extract was filtered through a small filter-paper (2 cm.) into a graduated test tube and 1 cc. conc. HCl was added to every 4 cc. of the extract. After the addition of a few drops of mercury the acid solution was reduced by magnesium powder, added gradually in small amounts until the maximum colour intensity was reached. After reduction the coloured extracts were mixed and made up to 50 or 100 cc. respectively with 90 per cent. alcohol.

It became at once obvious that the colour intensity of the reduced extract from the plants collected in the Alps was much stronger than that of the plants grown in the lowland, so much so that it became necessary to dilute it largely before comparison in the colorimeter. Estimations were made by means of Duboscq's and Hellige's colorimeters. Owing to the presence of small amounts of chlorophyll decomposition products, the colour of extract (2) could not be matched in Duboscq's apparatus, but the slight difference in tint did not interfere with the estimation in Hellige's wedge colorimeter. The results of many readings, in which the standard was set at different heights, were as follows:

Alpine Flowers	Flowers from London Plants			
	(1)	(2)	(3)	(4)
100	25·2	26·0	28·1	21·6

It will be seen therefore that the reduction colour, which serves as an index of the amount of flavone originally present, reaches on the average in London grown plants only 25 per cent. of that produced in the plants grown at higher altitudes.

Distribution of Flavone derivatives in Edelweiss plants. Whilst the flower-heads as a whole contained a large amount of flavone, it was found that the

stems of plants collected in the Alps contained much less and the stems of London grown plants were quite devoid of chromogenic substance. On the other hand the fruit (achene with its pappus) is extremely rich in it. Extracts of fruits and the corresponding flower bracts were compared quantitatively with the result that the reduction colour of the flower bracts was found to be a little more than half (54 per cent.) of that of the fruits. It seems therefore that the flavone derivatives are stored up in the seed and in accordance with this it was found that the inflorescence gathered in November had lost with its fruit the whole of its chromogenic substance. Vegetative leaves as well as roots of plants examined in December, were also devoid of chromogen.

The Flavone Contents of Edelweiss as a Biological Factor. The distinguishing feature of this plant, especially of its inflorescence, consists in its covering by long woolly hairs which give rise to the idea of whiteness expressed in its name. As an explanation for the biological significance of this pubescence it has been assumed that the air entangled between the matted hairs checks excessive transpiration and helps the plant to exist under very dry or desert conditions. The fact that under the influence of the moist English climate the plant continues through many generations to produce this characteristic feature is to some extent against this explanation, and together with the results of the above described observations tends to lend support to the suggestion of Shibata. The growing season of the alpine plants coincides with the maximum intensity of chemical sunlight in the year and the necessity of some protective mechanism of the living protoplasm against the injurious action of ultra-violet light is apparent. The flavones dissolved in the cell sap may well exert this protective action, as they do when examined in ordinary solution. The same plants grown in London under conditions in which the insolation is much less intense, apparently adjust themselves to the altered conditions by producing the protective substances in smaller amounts. In this connection it is interesting to note the behaviour of some other white alpine flowers which I had occasion to examine in February, a month in which the amount of ultra-violet light is extremely low. Extracts of the petals of *Saxifraga burseriana* and of *S. Boydii alba* (burseriana × aretioides) which flowered in my garden in the first half of February, gave practically no colour on reduction. On the other hand the white flowers of saxifrages (*S. cotyledon*, *S. longifolia*, *S. lingulata*, *S. Wallaceii*), examined later in the season, contained flavones. The petals of *Dryas octopetala* and *Campanula pusilla alba*, also gave intense reduction colours when examined in May and July respectively.

SUMMARY.

The inflorescence of Edelweiss (*Leontopodium alpinum*) contains a chromogenic substance, probably a flavone, which is not in glucosidic combination. Plants grown in London at an altitude of 80 m. contain only one fourth of the amount found in plants collected in the Alps at an average altitude of 2000 m. These facts illustrate the biochemical adaptation of alpine plants to changed environment and speak in favour of the assumption that the biological significance of flavones in the plant consists in their protective action against the injurious influence of ultra-violet light.

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XXVI. ON AMINO-ACIDS.

By HENRY DRYSDALE DAKIN.

(Report to the Medical Research Committee.)

(*Received August 22nd, 1918.*)

The following paper contains results of investigations on the separation, preparation and properties of various amino-acids and their derivatives. Some new methods have been made use of which promise to be of considerable value and have already led to the isolation of a new amino-acid and peptide. The complete working out of the experimental details will take a considerable time, but as certain of the results already obtained are of a definite character and the methods appear to have a wide applicability, it appears desirable to give an account of the work at the present time, leaving many obvious extensions as subjects for further study. The material here presented is divided into three main portions, which will be considered separately:

I. The extraction of amino-acids by means of partially miscible solvents.

II. Hydroxyglutamic acid, a new hydroxyamino-acid of the dicarboxylic acid group.

III. A new peptide, isoleucylvaline anhydride, obtained by the acid hydrolysis of caseinogen.

I. The extraction of amino-acids by means of partially miscible solvents.

The writer has long sought some means of extracting amino-acids from their aqueous solutions. Experiments were made with a variety of acid or basic solvents, in the hope of extracting basic or acidic amino-acids, but without success. Later experiments were made in which attempts were made to extract the strongly acid methylene amino-acid derivatives obtained by the action of formaldehyde, but the results were unsatisfactory. Recently, however, observations were made which led to a surprisingly simple method of extracting practically quantitatively many amino-acids from their aqueous solutions. For a particular purpose, the writer was anxious to obtain some

optically pure proline, and as the ester method invariably gives a largely racemised product, it was decided to attempt the direct extraction of proline from the products of hydrolysis of caseinogen by means of alcohols. The probability of success seemed small, since Fischer and Abderhalden [1904], in writing of the separation of proline from other amino-acids by means of alcohol, strongly emphasise the necessity of a previous distillation of the amino-acid esters. However, on hydrolysing gelatin with sulphuric acid and subsequently removing the latter as barium sulphate and then extracting the resulting concentrated solution of amino-acids with amyl alcohol in a continuous extractor, it was found that the proline was readily extracted. Soon after the extraction was started, the surprising observation was made that large quantities of solid amino-acids separated from the alcoholic extract and this seemed the more curious since they are practically insoluble in ordinary amyl alcohol. The phenomenon seemed sufficiently interesting to warrant further investigation and similar experiments were made with other alcohols. Propyl, butyl and isobutyl alcohols and various mixtures were used and it was found that butyl alcohol was the most satisfactory. This substance is now obtainable at nominal cost, owing to its large production in the fermentation process of acetone manufacture. Its boiling point, lower than that of iso-amyl alcohol, is a practical convenience and its rate of extraction is as good as that of any of the others.

On extracting amino-acid solutions from caseinogen and other proteins with butyl alcohol, it was found that the proline and practically the whole of the monamino-acids were extracted, together with some peptide anhydrides, which will be referred to later. The strongly ionised dibasic and dicarboxylic acids were left behind practically quantitatively. The only "neutral" monamino-acids detected in the extracted material have been minute traces of serine and in the case of amino-acids from gelatin the complete extraction of glycine seemed more difficult than that of the other amino-acids. In general, the extraction is surprisingly rapid and with the simple apparatus employed the extraction of two or three hundred grams of mixed amino-acids is complete in 36 hours. The bulk of the monamino-acids separate out in the extraction flask as a cream-coloured granular powder. It is convenient to filter off the monamino-acids occasionally during the extraction in order to avoid bumping or local overheating, using the mother liquor for continuing the extraction. The solid monamino-acids are washed first with a little butyl alcohol, then with a little ether, which removes traces of pigment and water. The butyl alcohol mother liquor contains the whole

of the proline, together with small amounts of amino-acids and peptide anhydrides, which can be largely separated through their sparing solubility in alcohol or water.

On examining the residue of non-extracted amino-acids, it was found to contain practically the whole of the diamino-acids, as judged by quantitative precipitation with phosphotungstic acid, and also the dibasic acids, aspartic acid, glutamic acid and a new dibasic acid to be described later. Larger yields of the dibasic acids were obtained from caseinogen than have previously been obtained by other methods. None of the dibasic acids could be detected among the extracted monamino-acids, so that it is clear that for practical purposes, they may be regarded as quantitatively separated from the latter.

It is thus seen, that by the use of butyl alcohol as a solvent, the products of hydrolysis of a protein may be readily separated almost completely into the following five groups:

(1) Monamino-acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol.

(2) Proline, soluble in alcohol and extracted by butyl alcohol.

(3) Peptide anhydrides (diketopiperazines) extracted by butyl alcohol, but separated from (2) by sparing solubility in alcohol or water.

(4) Dicarboxylic acids, not extracted by butyl alcohol.

(5) Diamino-acids, not extracted by butyl alcohol, but separable from (4) by phosphotungstic acid and other means.

Those who have had much experience in the separation of amino-acid mixtures will appreciate the following points. (*a*) The groups are composed of chemically similar individuals. (*b*) Practically the whole of the products of the hydrolysis of an amino-acid may be divided into the five groups without serious loss and each can be readily obtained in solid form, aliquot parts of which may be used in the search for individual acids. (*c*) No indications of any racemisation have been observed. All the amino-acids thus far separated possessed their full optical rotation, thus limiting the number of isomers in the various mixtures and permitting greater use of the polarimeter for identification purposes. The absence of racemisation has an obvious bearing on the use of the method for purposes of preparing material for bacterial and animal metabolism experiments. (*d*) Materially higher yields of many amino-acids may be obtained from proteins than by existing methods, thus permitting a more nearly quantitative analysis of the proteins themselves.

The apparent paradox of the extraction from aqueous solutions of monamino-acids, which are essentially insoluble in all alcohols, by means of butyl alcohol or similar compounds requires some reference. It soon became clear that the presence of water was a conditioning factor and that the passage of a certain proportion of water from the fluid undergoing extraction to the butyl alcohol medium, was essential. It was found that a concentration of amino-acids rapidly extractable by butyl alcohol yielded almost no amino-acids in the extract, if the aqueous phase contained an excess of salts such as calcium chloride. The obvious inference was that the passage of water from the aqueous to the butyl alcohol phase was impeded with corresponding reduction in the rate of extraction. Butyl alcohol at room temperature is miscible with about twelve parts of water, but I know of no experiments on the relative distribution of water and butyl alcohol when shaken together in excess. It can hardly be doubted that the successful extraction of monamino-acids from aqueous solutions by butyl alcohol is dependent both on the limited passage of butyl alcohol into the aqueous layer and of water into the alcohol layer. The conditions empirically found in the present case seem excellent for the purpose in view, but it would seem well worth while to consider the possibility of a much more extensive use of the present principle with other extractable substances and solvents. So far as I am aware, no systematic investigation of the possibilities of extractions by partially miscible solvents has been undertaken, though the common method of extraction of aqueous solutions with ether is in reality an extraction with ether plus water, and various substances, *e.g.* hydantoins, can be quantitatively extracted in this way from aqueous solutions, although they are almost insoluble in pure ether, and readily soluble in water. It can hardly be doubted that the use of butyl alcohol and similar solvents, will be found of value for many other purposes than the one referred to. It is not unlikely that certain sensitive substances may be selectively extractable from tissue extracts with a minimum of alteration, especially if the extraction be carried out under reduced pressure and hence at a reduced temperature. Tryptophan, formed from caseinogen by tryptic digestion, is readily extracted practically quantitatively in an almost pure condition after a single preliminary purification with mercuric sulphate. Another obvious application of the method is in the study of the products of the hydrolysis of proteins by enzymes.

Experimental.

The earlier experiments in which solvents other than butyl alcohol were used, will not be described, since the latter solvent appears preferable. Hydrolysed gelatin was used in many preliminary experiments, but as the results with caseinogen are rather more complete, they alone will be considered.

Hydrolysis. Purified caseinogen varying in amounts from 100–400 g. was hydrolysed by boiling for 12 to 16 hours, with five to six times its weight of diluted sulphuric acid prepared by mixing the concentrated acid with three volumes of water. Hydrolysis under these conditions is still incomplete, judged by the subsequent separation of peptide anhydrides in significant amount. After the heating was ended, the solution was diluted with water and the sulphuric acid quantitatively removed with barium hydroxide in the usual way. The filtrate, which still reacts acid to litmus but not to congo-red, was concentrated moderately and about 2 per cent. of crude tyrosine allowed to crystallise out. The filtrate was again concentrated and made approximately neutral to litmus by the further addition of barium hydroxide. The optimum reaction has still to be determined and probably varies with different proteins. In the case of gelatin feebly acid and alkaline reactions obtained by adding acetic acid or ammonia were successively tried, but this is not necessary with caseinogen and it is doubtful if it is ever desirable.

Extraction. The approximately neutral solution of amino-acids prepared as above described was concentrated on the water-bath until crude leucine began to separate and then the whole mixture was transferred whilst still warm to the extraction apparatus. Usually an apparatus of the simple Kutscher-Steudel type was employed, although others proved equally satisfactory. The amino-acids from 100 g. of caseinogen may be conveniently extracted in an apparatus holding about 350 cc. It is preferable to arrange conditions so that the aqueous layer occupies three-quarters or five-sixths of the available space and the volume of supernatant butyl alcohol is relatively small. It has been found convenient to substitute a rather larger extraction flask made of tough glass of the Jena type for the smaller flask usually employed. Rubber connections can be employed, although they are attacked by the hot alcohol to a considerable extent and their exposed surface should be reduced to a minimum. The extraction flask is heated over a sand-bath, preferably with rather high sides, so as to reduce condensation in the flask, and the flame is adjusted so that a reasonably rapid flow of alcohol returns

into the flask. When using a 300–400 cc. or smaller apparatus, the aqueous solution soon attains a temperature of from 60°–80° and neither cooling nor heating is required, but when larger volumes of fluid are being extracted, it is a good plan to warm it to 75 or 80° by means of a water-bath.

Soon after the extraction has begun, a separation of cream-coloured granular particles of amino-acids is observed in the extraction flask and the amount steadily increases. After extraction has continued during a working day, it will be found convenient to allow the extract to stand overnight and to filter off the separated amino-acids on a Buchner funnel, washing them with a little butyl alcohol and finally with a little ether. The combined alcoholic filtrates which are light brown in colour are used for continuing the extraction on the second day. Risk of breakage due to the accumulation of solid amino-acids on the bottom of the flask is thus avoided and it is possible to judge the completeness of the extraction by observing the increment of solid amino-acids on continued heating. When after several hours' extraction no significant additional amount of solid matter separates out of the butyl alcohol solution, the operation is regarded as finished. With the apparatus employed and extracting 1 to 400 g. of mixed amino-acids, the operation usually took about 36 hours; the time varying naturally with the rate at which the butyl alcohol is boiled.

When the extraction is over, the aqueous solution is reserved for examination for bases and dicarboxylic acids, as will be described later. The butyl alcohol extract, containing the proline and some peptide anhydrides, is filtered from the separated amino-acids, after standing at room temperature for some hours, while the monamino-acids, filtered and washed as previously described, are dried and weighed.

Butyl alcohol extract. Separation of proline, etc. The filtered extract is evaporated to dryness in a distillation flask under reduced pressure and the sticky brownish residue boiled with about ten parts of absolute alcohol. In the case of caseinogen at least, the whole of the residue is soluble in ethyl alcohol, indicating the absence of any considerable quantity of monamino-acids other than proline. On allowing the alcoholic solution to stand overnight, crystallisation occurs with separation of a small quantity, averaging 2 per cent. of the caseinogen, of an impure mixture chiefly composed of peptide anhydrides with traces of monamino-acids. This product was filtered off, washed with alcohol and examined later. The alcoholic filtrate was then evaporated under reduced pressure, dissolved in hot water, boiled with charcoal, filtered and concentrated to syrup.

A further crystallisation of peptide anhydrides occurs on standing, averaging 2 to 3 per cent. of the caseinogen taken, and this is filtered off and examined together with the similar product obtained above. The total yield of peptide anhydrides in different experiments was as follows: 2·7, 2·2, 3·2, 5·0 and 5·7. The larger yields were obtained from experiments with the larger amounts of caseinogen.

The clear aqueous solution after separation of the two fractions just described contains the whole of the proline. It is already much purer than might be anticipated from its method of preparation and compares favourably with the alcohol-extracted product obtained by the ester method.

It appears that the actual proline content can be closely estimated by means of van Slyke's methods for differentiating between amino and imino nitrogen, for a characteristic derivative of proline was isolated in amount corresponding closely to that indicated by analysis. It was found that no purification was effected by converting the proline into its copper salt for the whole of the product was soluble in methyl alcohol. It will be convenient to give the yields of proline as indicated by van Slyke's differential analysis and then to refer to a new derivative of proline which seems useful for purposes of characterisation.

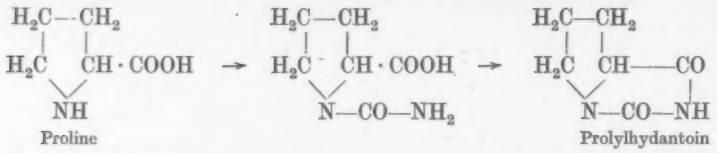
Caseinogen analysed	Nitrogen of Proline Fraction		Percentage Proline calculated
	Total	Non-Amino	
150 g.	1·70 g.	1·34	7·3
64	0·683	0·557	7·1
50	0·611	0·521	8·5
18	0·224	0·184	8·2
232	2·94	2·45	8·7

The average of the above determinations is close to 8·0 per cent. proline and evidence other than analytical will be presented to show that no considerable amount of other substance than proline is contained in the above estimate. It is of interest to compare the above figures for the proline content of caseinogen with that obtained by other workers. Van Slyke [1911], using his analytical methods on amino-acids separated by the ester method, obtained 6·70 per cent., while his analysis of caseinogen by direct hydrolysis indicated that 7·13 per cent. of the total nitrogen of caseinogen was in the non-amino form. If the whole of this were due to proline, ignoring the small amount of hydroxyproline it would be equivalent to 9·2 per cent. proline. Abderhalden obtained only 3·1 per cent. of proline by the ester method, while Foreman in unpublished experiments is quoted by Plimmer [1917] as having obtained 7·6 per cent., a figure in close agreement with that found by me.

By the direct extraction method just described, there is no evidence of any racemisation of the proline as judged by the solubility of copper salts in methyl alcohol and by the preparation of other derivatives, but on the other hand, the impurities which accompany the proline appear to be fairly strongly dextro-rotatory, so that a direct polarimetric estimation of proline is not possible. The observed rotations correspond to about three-fourths of the proline believed to be actually present.

Preparation of l-prolylhydantoin. The preparation of uramido-acids and the hydantoins derived from them by the action of mineral acids has not been made as much use of for the identification and separation of amino-acids as the method deserves. In many cases the products are more easily purified than the more generally known phenyl derivatives, obtained by the use of phenyl isocyanate, on account of well-marked solubility differences. Proline affords a good example of the utility of the method. On warming crude proline on the water bath with a moderate excess of potassium cyanate, it is converted smoothly into the uramido-acid, and the same is true of any contaminating amino-acids. On liberating the uramido-acids by making the solution just decidedly acid to congo-red and extracting with ether for a few hours in a continuous extractor, practically none of the proline compound is extracted, but the whole of any leucine, isoleucine, valine, alanine, phenylalanine and tyrosine derivatives are removed. On heating the extracted aqueous solution with 10–20 per cent. sulphuric or hydrochloric acid for an hour and then re-extracting with ether, prolylhydantoin is obtained in excellent yield and possesses convenient properties for purposes of identification. The following gives details of a typical experiment.

Crude *l*-proline (5 g.), containing as judged by van Slyke's analytical methods about 16 per cent. of impurity, was evaporated on the water bath with potassium cyanate (5 g.) and water (50 cc.). On taking up with water, acidifying to congo-red with sulphuric acid and extracting with ether for seven hours, 0·55 g. of uramido-acids, chiefly derived from valine and leucine, were obtained. The extracted aqueous solution was then heated for 1–2 hours on the water-bath after the addition of one-fifth volume of 50 per cent. sulphuric acid and then again extracted with ether for 12 hours. On evaporation of the ether 4·3 g. prolylhydantoin was obtained in the form of transparent shining thick prismatic needles. The substance is very easily soluble in hot water and readily crystallises from the concentrated solution. The yield of pure products amounted to about 85 per cent. of the theoretical amount, after allowing for the known impurity in the crude proline.



l-Prolylhydantoin has a high optical rotation, as shown by the following observations on two different preparations:

(1) $c = 2{\cdot}0$, $l = 1{\cdot}0$, $\alpha = -4{\cdot}77°$ hence $[\alpha]_D^{20°} = -238{\cdot}5°$,

(2) $c = 2{\cdot}0$, $l = 1{\cdot}0$, $\alpha = -4{\cdot}64°$ hence $[\alpha]_D^{20°} = -232{\cdot}0°$.

On analysis, the two specimens were found to contain 19·80 and 19·95 per cent. nitrogen (Kjeldahl) compared with a calculated value of 20·0 per cent. *l*-Prolylhydantoin, after recrystallisation from water, melts at 165–167°. When prepared from partially racemised proline obtained by the ester method, the melting point is materially lower, *e.g.* 153–156°.

It is not without interest to note that *l*-prolylhydantoin is racemised by alkali at room temperatures much less readily than most other hydantoins observed by the writer. A 1 per cent. solution in 0·4 *N* NaHO still possessed about one-third of its initial rotation at the end of a week. The writer has advanced the view that racemisation of hydantoins by alkali at low temperatures is due to keto-enol tautomeric changes and as the CH group adjacent to the CO in prolylhydantoin, unlike the other hydantoins examined, forms part of the cyclic structure, it is not improbable that this fact is related to its slower rate of racemisation.

It is believed that *l*-prolylhydantoin may serve as a useful derivative for the characterisation of proline in impure mixtures, when successively converted into uramido-acid and hydantoin. It has been already stated that under these conditions, contaminating alanine, valine, leucine, isoleucine, phenylalanine and tyrosine are removed as uramido-acids on extraction with ether. On the other hand, glycine, serine, proline, hydroxyproline, aspartic and glutamic acids, are not extracted by ether as uramido-acids, but are extracted after conversion into their hydantoins. The practical utility of these facts under various conditions, is sufficiently obvious and will be studied in further detail.

The monamino-acids extracted by butyl alcohol.

The amino-acids extracted by butyl alcohol other than proline, separate out as previously described as a cream-coloured granular deposit. They dissolve in water to give a clear light yellow solution and contain only insig-

nificant traces or none of the diamino-acids or dibasic acids. The reaction of the aqueous solution is barely acid to litmus.

The yield of these monamino-acids from caseinogen is indicated as follows:

48 g. caseinogen gave 18 g. dry monamino-acids = 37·5 per cent.
150 „ „ „ 51 „ „ „ = 34·0 „
232 „ „ „ 86 „ „ „ = 37·3 „

The above figures do not include about 2–3 per cent. of tyrosine filtered off prior to extraction, so that the total yield is approximately 40 per cent. of the caseinogen taken. It is not unlikely that slightly higher figures might follow greater care in avoiding loss in the removal of sulphuric acid following hydrolysis. The amino-acids thus separated contain about 11·5 per cent. of nitrogen and are chiefly made up of alanine, valine, leucines and phenylalanine, with a little tyrosine. Hydroxyproline and serine appear to be present, but the complete analysis of the mixture awaits further investigation. The product is in a most convenient form for application of the ester method, but it is believed probable that preliminary separations can be advantageously made. Apart from the traces of valine and leucine which accompany the crude proline, it is believed that the whole of these acids are contained in the fraction under consideration and most careful search by various methods has failed to detect them in the other fractions, provided the preliminary extraction with butyl alcohol has been adequate. Glycine, which appears to be absent or only present in traces in caseinogen, is only extracted quantitatively with difficulty, as experiments with gelatin showed. Traces of serine have also been detected among the unextracted amino-acids. A further report on the composition of this monamino fraction will be made at an early date.

Experiments on the rate of extraction by butyl alcohol of individual amino-acids from pure aqueous solution will also be made.

It is perhaps not out of place to refer to the possible use of such a readily obtainable dry, almost neutral, amino-acid mixture for furnishing a basis for nutrient media, with or without the addition of tryptophan. The fact that most of the amino-acids which furnish glucose in the diabetic organism are absent from this material suggests dietetic possibilities.

The amino-acids not extracted by butyl alcohol. Separation of aspartic, glutamic and other acids.

As has already been stated, the amino-acids which are not extracted by butyl alcohol from aqueous solution, are the strongly ionised diamino-acids and dicarboxylic acids. Quantitative experiments, in which the mixture was

precipitated with phosphotungstic acid, showed that the whole of the diamino-acids could be recovered in this way, but the filtrate yielded less of the dicarboxylic acids than could be obtained by other methods, so that for purposes of quantitative analysis of proteins, it is decidedly preferable to use an aliquot portion for the examination of the bases and the rest for the dicarboxylic acids.

The diamino-acids. The precipitation with phosphotungstic acid and subsequent separation of histidine, arginine and lysine requires no comment other than the desirability of removing dissolved butyl alcohol from the solution by heating before precipitation, for the inhibitory action of alcohols on the precipitation is well known.

The dicarboxylic acids. The degree of purification effected by the removal of the mono-amino acids by extraction, permits a much more nearly quantitative separation of glutamic acid as hydrochloride than is usually the case. Precipitation moreover is usually more rapid and after removing the first crop of crystals, concentrating and re-saturating with hydrogen chloride in the usual way, the second crop of crystals is very small. It may be noted here that in the analysis of gelatin and other proteins containing much glycine, the precipitation of glycine hydrochloride along with the glutamic acid hydrochloride is possible on long standing in concentrated solution. Caseinogen gave the following yields of glutamic acid hydrochloride. In each case, the glutamic acid hydrochloride was filtered off on asbestos and well washed with cold hydrochloric acid. It was then dissolved in a small volume of water and again precipitated by saturation with hydrogen chloride. The only impurities to be feared were ammonium chloride and barium chloride and these were excluded by controlling the purity of the hydrochloride by analysis, using the Kjeldahl or van Slyke methods, after evaporation of the portion used for analysis with calcined magnesia. The values obtained by either method were all within 0·1 per cent. of the theoretical value of 7·65 per cent. of nitrogen.

I.	16 g.	caseinogen	gave	4·3 g.	glutamic	acid	hydrochloride	= 21·6 %	glutamic	acid
II.	50 „	„	„	13·2 „	„	„	„	= 21·2 „	„	„
III.	150 „	„	„	36·7 „	„	„	„	= 19·6 „	„	„
IV.	232 „	„	„	42·6 „	„	„	„	= 14·7 „	„	„

The first three experiments quoted above gave reasonably concordant results indicating an average of about 21 per cent. of glutamic acid. The last experiment, IV, differed from the others in that previous precipitation of the diamino-acids with phosphotungstic acid was carried out and the filtrate was then freed from sulphuric and phosphotungstic acids by means of barium

hydroxide. There was undoubtedly considerable loss connected with the handling of the bulky precipitates, and it seems reasonable to exclude this low result in computing the average. The proportion of glutamic acid in caseinogen, as estimated above, is materially higher than that previously recorded, with the exception of Foreman's estimations [1914]. Foreman obtained about 17 per cent. of glutamic acid as such, together with pyrollidone-carboxylic acid, determined analytically and presumably derived from glutamic acid, indicating a total yield of 21·8 per cent. glutamic acid. These figures are in excellent agreement with those now presented and are six to 12 per cent. higher than those previously recorded.

The next problem was the separation of aspartic acid from the filtrate from the glutamic acid hydrochloride. Various obvious methods were tried with unsatisfactory results, partly due to the presence of another dicarboxylic acid, to be described in the following section. Levene and van Slyke [1910] showed that aspartic acid was almost completely precipitated on boiling its aqueous solution with excess of lead oxide, the apparent solubility of the salt being about 1 in 4700. This fact was utilised successfully for the separation of aspartic acid as follows. The filtrate from the glutamic acid hydrochloride was concentrated under reduced pressure to remove as much of the hydrochloric acid as possible and the resulting syrup dissolved in about ten parts of hot water. It was then treated with calcium hydroxide and the calcium salts precipitated by alcohol as described by Foreman [1914]. The sticky deliquescent calcium salts after washing with alcohol were dissolved in water and the calcium removed exactly with oxalic acid. Freshly precipitated and well washed lead hydroxide was then added by degrees while the solution was kept boiling under a reflux condenser. The addition of lead hydroxide was continued until the reaction of the solution was distinctly alkaline to litmus and heating was then continued for a further 15 minutes. The mixture was then allowed to cool and stand overnight. The precipitate containing the aspartic acid and much lead chloride was filtered off and washed with cold water; the filtrate being reserved for the separation of the acid to be described in the following paper. The lead precipitate is somewhat difficult to decompose with hydrogen sulphide, so that it is more convenient to boil it with dilute sulphuric acid in moderate excess. Almost all the lead is thus removed and the filtrate contains aspartic, hydrochloric and sulphuric acids. The filtrate is then concentrated with excess of precipitated barium carbonate, filtered and made up to a convenient volume such as 1 cc. per gram of protein taken. A nitrogen estimation on a small aliquot part gave an indication of

the possible aspartic acid concentration. A hot concentrated solution of normal copper acetate was then added in amount equal to 1·5 molecules per molecule of aspartic acid. On allowing the solution to stand for a couple of days in a cool place, the characteristic crystalline copper aspartate separated out almost completely and was filtered off, washed, dried and weighed.

In two experiments, 150 g. of caseinogen gave 9·7 and 11·6 g. of crystalline analytically pure copper aspartate. The salt, after drying in the air, contained more nearly 3 mols. of water than the more customary $4\frac{1}{2}$, similar observations having been made by Curtius and Koch and by Skraup. The anhydrous salt dried at 130° contained 32·6 per cent. copper and 7·2 per cent. nitrogen, compared with calculated values 32·7 and 7·2 respectively.

The yields of crystalline copper aspartate noted above correspond to 3·5 and 4·1 per cent. aspartic acid respectively, in caseinogen, and are two to three times as large as the figures previously recorded.

The extraction of tryptophan by means of butyl alcohol.

The hydrolysis of caseinogen by sulphuric acid completely destroys the tryptophan, as is well known, and none of it was encountered in the preceding separations of the products of acid hydrolysis. It appeared desirable to determine whether tryptophan if present in a solution could be extracted, and accordingly the following experiment was made.

Caseinogen (187 g.) in two litres of 1 per cent. sodium carbonate solution was digested for seven days with active pancreas extract. The solution was then treated as described by Hopkins and Cole [1902] and precipitated *once* with mercuric sulphate solution. The precipitate was decomposed with hydrogen sulphide, sulphuric acid removed exactly with barium hydroxide and the solution concentrated to about 30 cc. under diminished pressure. It was then extracted with butyl alcohol in a small extractor. A separation of tryptophan on the sides of the extraction flask was soon noted, and at the end of two hours' extraction 1·5 g. had separated out. The extraction was practically quantitative at the end of 12 hours and the residual aqueous solution gave only a doubtful reaction with Hopkins' sensitive glyoxylic acid reaction. The tryptophan crystallising from the butyl alcohol extract weighed 3·15 g. and was filtered off and washed with ether. The mother liquor (50 cc.) on evaporation gave a sticky residue mostly soluble in hot absolute alcohol and containing but a few centigrams of tryptophan.

The main portion of tryptophan (3·15 g.), corresponding to a yield of 1·7 per cent. on the caseinogen was remarkably pure, considering its simple

method of separation. Apart from a little yellow pigment and a minute trace of tyrosine it seemed to be pure, as judged by its melting point, optical rotation and analysis.

A 1 per cent. solution, without needing clarification, showed a rotation of $-0{\cdot}68°$ in a 2 dm. tube, hence $[\alpha]_D = -34°$. The recorded values vary from -30 to $-40°$, but the above figure agrees well with Hopkins and Cole's estimate of $-33°$. On analysis (Kjeldahl) 13·9 per cent. nitrogen was found, compared with 13·72 per cent. calculated.

The preparation of tryptophan in a pure state in considerable quantities is an operation requiring some skill and experience in order to avoid loss of much of the product in mother liquors which crystallise with some difficulty. The extraction method it is believed will simplify the process to some extent and probably requires less careful manipulation. The crude product is already fairly pure and a single recrystallisation from dilute alcohol, as recommended by Hopkins, furnishes a completely pure substance. The method could probably be operated on a considerable scale and may be of service in obtaining tryptophan for bacteriological and other purposes.

II. A new Dibasic Amino-Acid occurring in Proteins.

It was found that after the separation of glutamic and aspartic acid as described above, large amounts of at least one other dicarboxylic acid were still present and could be isolated.

For simplicity in presentation, it is convenient to anticipate the experimental portion of the paper and to proceed at once to the main properties and probable constitution of the new acid.

(1) The acid is optically active and is extremely readily soluble in water, crystallising slowly in thick prisms from its syrupy solution. Most of its metallic salts, with the exception of those of silver and mercury, are also very soluble in water and do not crystallise readily but may be precipitated by alcohol. It forms a diethyl ester which has not yet been satisfactorily distilled without decomposition.

(2) The acid itself appears to possess the formula $C_5H_9O_5N$ and the salts of the heavy metals contain two equivalents of bases, while salts of the alkaline earths contain one.

(3) The acid behaves as a strong monobasic acid on titration with sodium hydroxide, using phenolphthalein as indicator and the results are in accord with a molecular weight of 163. The same molecular weight follows from the analysis of the well characterised silver salt, $C_5H_7O_5NAg_2$. In the presence of

excess of formaldehyde, the substance behaves towards sodium hydroxide as a dibasic acid.

(4) The acid, if not heated too strongly, contains the whole of its nitrogen in the (NH_2) form, as judged by its reaction towards nitrous acid. On prolonged heating over phosphorus pentoxide at 100–110°, a molecule of water is slowly eliminated and the nitrogen is converted into the (NH) form. This reaction is clearly analogous to the formation of pyrollidonecarboxylic acid from glutamic acid and is evidence of one carboxyl group being in the γ position relative to the amino-group.

(5) The acid or its salts on ignition give a definite but faint pyrrole reaction, especially if much charring occurs, thus favouring reduction. On the other hand, on heating with zinc dust, an intense pyrrole reaction is immediately obtained.

(6) On reduction with fuming hydriodic acid at 150°, glutamic acid and other products are obtained.

(7) The acid gives characteristic colour reactions with various phenols and concentrated sulphuric acid resembling aliphatic hydroxy-acids.

(8) On oxidation of the sodium salt of the acid (1 mol.) with chloramine-T (1 mol.) an aldehyde is obtained, presumably $C_4H_6O_4$, which on treatment with *p*-nitrophenylhydrazine gives a characteristic *osazone* containing one carboxyl group, the analysis of which agrees well with the formula $C_3H_3COOH(:N.NH.C_6H_4NO_2)_2$. The formation of the osazone from the aldehyde-acid is indicative of a hydroxyl group adjacent to the aldehyde group and hence contiguous to the amino-group in the original acid[1].

(9) No indication of lactone formation was observed, thus confirming the assumed position of the hydroxyl group as noted under (8) and excluding a γ position.

(10) On treatment with potassium cyanate a uramido-acid is formed which is not extractable from aqueous solution by ether. The uramido-acid, on heating with acids, gives an extremely soluble hydantoin, which is extractable by ether, thus resembling similar derivatives of aspartic and glutamic acid.

(11) The acid gives derivatives with phenyl isocyanate and β-naphthalene-sulphonic chloride, but they are not well adapted for purposes of identification.

[1] On oxidising the sodium salt with two molecules of chloramine-T, under the conditions which the writer used to convert glutamic acid into β-cyanopropionic acid [1917], a substance was obtained which on hydrolysis apparently furnished *malic acid*. The latter acid has only been identified thus far as calcium salt and by colour reactions. The reaction will be studied further.

The above results can hardly be reconciled with any other structure for the acid than that of an α-amino-β-hydroxyglutaric acid, *i.e.* β-hydroxyglutamic acid, $COOH . CH(NH_2) . CH(OH) . CH_2 . COOH$.

An acid of the above structure containing two dissimilar asymmetric carbon atoms can exist in a variety of forms and it is not at all improbable that the substance isolated by me consists of more than one individual. It will be a matter of some difficulty to determine this as the melting point of the acid appears not to be sharp, partly owing to its rapid conversion into hydroxypyrrolidonecarboxylic acid, and its satisfactory recrystallisation has practical difficulties. Recently, however, a series of beautifully crystalline salts of the acid with strychnine, brucine and other alkaloids have been obtained and a further study of these products may prove of service. The formation of an unsaturated acid, $COOH . CH(NH_2) . CH = CH . COOH$, under the influence of alkaline or acid reagents, is obviously possible and such a product can be reconverted into the β-hydroxy-acid with partial loss of asymmetry. A study of the configuration and synthesis of the new acid and some of its biochemical relationships will be continued.

The amount of the new acid in caseinogen is very considerable, for no less than 10·5 g. of an almost wholly crystalline mass has been obtained from 100 g. of caseinogen after hydrolysis by sulphuric acid[1]. Hydrolysis of caseinogen by barium hydroxide in an autoclave at 140°, gave only about 1·5 per cent. of a syrup crystallising with difficulty but apparently containing some of the acid.

An acid of the structure indicated above has a number of interesting biochemical relationships which invite speculation. The formation from it by oxidation *in vivo* of malic and perhaps tartaric acid is clearly possible. Its relationship with serine is obvious and if it should be shown that the new acid may be formed *in vivo* by the oxidation of glutamic acid, an interesting parallelism with the known β-oxidation of fatty acids would be established. The writer [1913] has already drawn attention to the probable similarity in the behaviour in the animal body of glutamic acid and proline and the existence of β- or γ-hydroxyproline and β-hydroxyglutamic acid as protein constituents may well give a clue to the intermediary metabolism of the former. It is interesting in this connection to recall the old observation of Buisine showing that malic acid comprises 2–5 per cent. of the water-soluble matter of sheep

[1] Osborne has drawn attention to the possible occurrence of hitherto unknown dicarboxylic acids in gliadin and glutenin owing to their disproportionately high yield of ammonia on hydrolysis, compared with their yield of aspartic and glutamic acids. These proteins will be examined for the new acid.

sweat. It appears probable that this may originate from β-hydroxyglutamic acid. The biochemical interconversion of malic acid, lactic acid and glucose is well established. The easy formation of cyclic hydroxypyrrolidonecarboxylic acid from β-hydroxyglutamic acid may also be significant in connection with the synthesis of the pyrrolidine acids *in vivo*. But further knowledge of the new acid is clearly more desirable than premature speculation.

The writer believes that the substance under discussion is probably the first example of a hydroxy-dicarboxylic amino-acid occurring among the proteins. Reference must, however, be made to Skraup's statement as to the occurrence of hydroxyaspartic acid in caseinogen. As Fischer has pointed out, Skraup's experiments are not described in a form capable of repetition and no evidence, other than the analysis of a copper salt, is adduced that the minute amount of substance obtained by Skraup was a dicarboxylic acid. The value of this analysis for determining the basicity of the acid is small, since no carbon and hydrogen determinations were made on the free acid. If hydroxyaspartic acid, as described by Skraup, were obtainable from caseinogen, it would almost surely have accompanied the β-hydroxyglutamic acid, but no such acid was detected. Moreover, a search for tartaric or racemic acids among the products of the action of nitrous acid upon the dicarboxylic acids of caseinogen gave negative results, thus indicating the probable absence of hydroxyaspartic acid.

On the other hand, certain experiments carried out in Hopkins' laboratory, at Cambridge, by F. W. Foreman, read in the light of later work clearly indicate that a partial separation of the new acid had been accomplished. Foreman made use of the insolubility of the calcium salts of dibasic amino-acids in alcohol for their separation—a method which the writer has since utilised in a modified form. He obtained from the calcium salts crystalline aspartic and glutamic acid and a syrup soluble in acetic acid but difficult to crystallise, which contained pyrrolidonecarboxylic acid and "other unidentified substances probably of great importance." While the writer's experiments originated from a totally different direction and were not influenced by Foreman's observations, there is little doubt that if the latter had been able to pursue his investigations uninterruptedly, he would have isolated β-hydroxyglutamic acid.

Experimental.

Preparation of β-hydroxyglutamic acid. Four separate preparations of the acid have been made by different methods, and in each case a substance with

similar properties was obtained. It is not proposed to describe the earlier less satisfactory methods, but simply to give an account of the method which hitherto has given the best result.

The material used was hydrolysed caseinogen thoroughly extracted with butyl alcohol in order to remove the monamino-monocarboxylic acids as described in the preceding section. Glutamic acid was next removed as completely as possible as hydrochloride by saturation with hydrogen chloride, taking care to resaturate the concentrated filtrate from the first crop of crystals and allowing the fluid to stand in an ice box for three days. The filtrate from the glutamic acid hydrochloride was then concentrated under reduced pressure, to remove as much hydrochloric acid as possible. The residual syrup was dissolved in water, digested with an excess of calcium hydroxide, filtered, concentrated to a syrup and then the calcium salts of the remaining dibasic acids were precipitated by alcohol. The calcium salts were then dissolved in water, the calcium removed with oxalic acid and the filtrate boiled with lead hydroxide to remove aspartic acid. The conduct of the preceding operations will be familiar to anyone accustomed to working with the amino-acids and are given in greater detail in the preceding section.

The filtrate from the lead aspartate (from 150 g. caseinogen) was freed from lead with hydrogen sulphide, diluted to 500 cc., acidified with 20 cc. of sulphuric acid and carefully precipitated with phosphotungstic acid to remove bases which are invariably present. The filtrate was then freed from sulphuric and phosphotungstic acids with barium hydroxide in the usual way and the excess of the latter removed fairly exactly with sulphuric acid. The volume of the filtrate, which was strongly acid, was brought to about half a litre. In the earlier experiments the new acid was precipitated with mercuric acetate and sodium carbonate, but later it was found that the silver salt was preferable.

Small amounts of hydrochloric acid are invariably present in the solution and this was first removed by adding dilute nitric acid (5 cc.) and then silver nitrate until no more precipitation occurred. The filtrate from the silver chloride was then made neutral or feebly alkaline with sodium hydroxide. The new acid was then precipitated by the alternate addition of strong silver nitrate solution (15 per cent.) and approximately normal sodium hydroxide. A heavy white precipitate was soon produced in the solution and more silver nitrate or sodium hydroxide was added as long as addition of either solution produced a white precipitate. The sodium hydroxide is best added by degrees from a burette and a large excess must be avoided since it decomposes the

precipitate. When excess of silver nitrate was present and precipitation completed, a test portion of the filtrate, on adding a drop of sodium hydroxide solution, gave only brown silver oxide. The end point is easily determined with a little care. The precipitated silver salt, which at first was pure white but on completion of the operation was light brown, from a little silver oxide, was filtered off using suction and washed with cold water. It was then decomposed with hydrogen sulphide and the filtrate concentrated under reduced pressure. It is probably advisable to avoid heating the solution unduly in order to limit any formation of hydroxypyrrolidonecarboxylic acid. A clear yellowish syrup containing the acid was obtained and the separation of crystals only commenced after most of the water had been removed in the vacuum desiccator. Gradually thick prisms begin to separate and eventually the whole is transformed into a solid mass of crystals. The crystalline magma from 150 g. of caseinogen weighed 19·5 g. and contained, as judged by titration with standard alkali and phenolphthalein, 17·5 g. of the acid. The acid is difficult to crystallise so that a convenient amount of mother liquor may be removed and a fairly good plan is to crush the crystals separated from water with a mixture of one part of glacial acetic acid and four parts methyl alcohol and finally to wash with methyl alcohol. Precipitation of the aqueous solution with alcohol gives a syrup which rapidly solidifies, and the same is true on adding alcohol to the concentrated acetic acid solution. The acid is extremely soluble in water, dissolving in about its own weight of water, and is readily soluble in acetic acid, slightly soluble in methyl alcohol but practically insoluble in ethyl alcohol, ether, or ethyl acetate. In aqueous solution the substance shows a low dextro-rotation which is increased on addition of hydrochloric acid. Its optical properties will be examined more carefully and reported later, as variation in concentration appears to influence the specific rotation considerably.

For analysis, the acid was dried in vacuo over phosphorus pentoxide at a temperature not above 40°:

0·1298 g.; 0·1741 g. CO_2, 0·0661 g. H_2O

0·2314 g. neutralised 13·9 cc. *N*/10 NaOH (Kjeldahl)

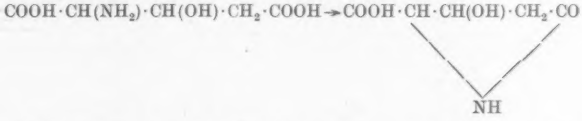
0·0195 g. gave 3·00 cc. N at 23° and 770 mm. (van Slyke)

	C	H	N	
Found	36·6	5·66	8·40	8·45 (van Slyke)
Calculated for $C_5H_9O_5N$	36·8	5·52	8·59	

Other analyses on less pure material gave results varying from 35·8–37·7 per cent. C, 5·6–6·2 per cent. H, 8·4–9·3 per cent. N.

Molecular Weight. 0·2071 g. of the acid dissolved in water and titrated with *N*/10 NaOH required 11·6 cc. when sensitive litmus paper was used as indicator and 12·3 cc. with phenolphthalein. Assuming that the acid reacts like glutamic acid as a monobasic acid, these figures correspond to molecular weights of 171 and 160 respectively, with a mean of 166. Another specimen of the acid (0·240 g.) required 14·2 cc. of alkali using litmus as indicator, equivalent to a molecular weight of 168. Analysis of the silver salt (see later) gave 57·3 per cent. Ag corresponding to a molecular weight of 163. The calculated value for $C_5H_9O_5N$ is 163.

The behaviour of the acid on heating is peculiar. In the neighbourhood of 100° the acid becomes pasty and appears to part with water. Eventually on raising the temperature the whole of the acid is converted into a clear glassy mass. The change is brought about rapidly at a temperature of about 140–150°, but can be effected in a few hours by heating over phosphorus pentoxide at 110°. Most of the nitrogen in the glassy compound, as already stated, is no longer in the (NH_2) form and no longer reacts with formaldehyde or nitrous acid. A sample of the product heated for 6 hours at 110° gave the following result on analysis: C 39·5 per cent., H 5·7 per cent., total N 9·4 per cent., amino-N 4·3 per cent. The results are obviously explainable on the basis of a mixture of β-hydroxyglutamic acid and hydroxypyrrolidone-carboxylic acid. The latter acid contains 41·3 per cent. C, 4·9 per cent. H, 9·86 per cent. total N and no (NH_2) nitrogen.



Silver Salt. This salt was used for the separation of the acid. A specimen for analysis was prepared, as already described, by adding silver nitrate (2 mols.) to the sodium salt of the acid (1 mol.) in about 5 per cent. solution. It separated as a granular very sparingly soluble pure white heavy precipitate showing crystalline structure on standing. It was filtered off and washed with water, alcohol and ether and dried *in vacuo* in the dark. The salt is not excessively sensitive to light. It was analysed with the following result:

0·2568 g.; 0·1495 g. CO_2; 0·0440 g. H_2O

0·1415 g. neutralised 3·6 cc. *N*/10 acid (Kjeldahl)

0·2976 g.; 0·1707 g. Ag.

	C	H	N	Ag.
Found	15·8	1·90	3·56	57·3
Calculated for $C_5H_7O_5NAg_2$	15·9	1·85	3·70	57·3

Copper Salt. The acid forms an extremely soluble copper salt on boiling with precipitated copper oxide and this fact was utilised in preliminary experiments, in order to be assured of the absence of aspartic, glutamic and other acids which form sparingly soluble copper salts. None of these latter salts was ever detected as an impurity in the new acid, and on the whole, the copper salt, for purposes of purification, is much less satisfactory than the silver salt. To prepare the copper salt, the acid was boiled in 2 per cent. solution with a large excess of well washed copper oxide for an hour under a reflux condenser. On filtering and concentrating, the syrup slowly solidified, giving some crystalline product which was hard to separate. It was found more convenient to add methyl alcohol by degrees to a fairly strong solution of the salt, stirring during the addition. The bulk of the copper salt is precipitated as a clear blue powder and a few crystals are apt to separate from the mother liquor on standing. A more complete precipitation is effected by ethyl alcohol, but methyl alcohol is preferred as the copper salt is slightly soluble in it if a little water is present and possibly a certain amount of purification may be so effected. The salt was dried at 110° and contained only traces of water. It was analysed with the following result:

0·1759 g.; 0·1681 g. CO_2; 0·0533 g. H_2O
0·1093 g.; 0·0379 g. CuO
0·1265 g. neutralised 5·8 cc. *N*/10 acid (Kjeldahl)
0·0120 g.; 1·31 cc. N. at 23° and 764 mm. (van Slyke).

	C	H	N	Cu
Found	26·1	3·36	6·42, 6·15	27·7
Calculated for $C_5H_7O_5NCu$	26·7	3·12	6·23	28·3

Mercury Salts. Mercuric chloride added to a solution of the acid produces no precipitate, but on adding excess of sodium acetate, a microcrystalline salt soluble in hot water is precipitated. It has not yet been analysed. The alternate addition of mercuric acetate and sodium carbonate, as practised by Neuberg and Kerb, precipitates the acid practically quantitatively, as a white amorphous salt of complex composition, and use has been made of this property for the separation of the acid. Mercurous salts also precipitate neutral solutions of the acid.

Lead Salt. The lead salt was prepared by boiling the acid in 2 per cent. aqueous solution for one hour with excess of well washed freshly precipitated lead hydroxide. The filtered and concentrated solution did not readily crystallise but dried up to a heavy clear glassy crystalline mass easily soluble in

water but insoluble in alcohol. A sample, dissolved in water and precipitated with alcohol, was obtained as a heavy white granular powder.

0·1350 g.; 0·0824 g. PbO.

Found ... ... 56·6 per cent. Pb

Calculated for $C_5H_7O_5N$ Pb 56·3 ,,

Cadmium and Zinc Salts. These salts were prepared in the same fashion as the lead salt. Neither of them crystallises readily and they are extremely soluble in water but insoluble in alcohol. They appear to be normal salts of the type $C_5H_7O_5N$ M″.

Calcium Salt. The free acid was warmed with excess of lime water and filtered. On concentrating on the water bath calcium carbonate and hydroxide separated out and the operation of filtration and evaporation was twice repeated. It appears that a normal calcium salt is first formed which decomposes on exposure to air to give the acid salt. The concentrated solution of the calcium salt refused to crystallise, and it was therefore precipitated with alcohol. The soft amorphous precipitate soon became hard and on drying *in vacuo* at 110° the substance became crystalline resembling fused sodium acetate. It is extremely soluble in water, insoluble in alcohol.

0·1098 g.; 0·0428 g. $CaSO_4$.

Found ... ... 11·5 per cent. Ca

Calculated for $(C_5H_8O_5N)_2Ca$ 11·0 ,,

Barium Salt. The acid barium salt was obtained by boiling the acid with excess of precipitated barium carbonate. The filtered solution on concentrating dries to a hard gritty mass. The salt is easily soluble in water, insoluble in alcohol. It was dried at 110°.

0·2241 g.; 0·1112 g. $BaSO_4$.

Found ... ... 29·2 per cent. Ba.

Calculated for $(C_5H_8NO_5)_2Ba$ 29·7 ,,

β-Naphthalenesulphonic derivative. One gram of the acid was dissolved in 6 cc. of normal sodium hydroxide and shaken with β-naphthalenesulphonic chloride (2·7 g.) dissolved in ether (15 cc.). Additional sodium hydroxide (30 cc.) was added by degrees with vigorous shaking. The mixture was allowed to stand overnight, and the aqueous portion filtered and precipitated with concentrated hydrochloric acid (6 cc.). A clear oil separated which became sticky on washing with water, and on long standing eventually solidified to a hard crystalline mass. It is sparingly soluble in water but readily soluble in alcohol and acetone, but all attempts to crystallise it satisfactorily failed. It was purified to some extent by redissolving in *N* NaOH

and again precipitating with acid. The substance was found to contain 4·04 per cent. of nitrogen (Kjeldahl) compared with a calculated value of 3·96.

On dissolving the naphthalenesulphonic derivative in a minimum (1 mol.) of warm *N*/10 NaOH and adding copper acetate, a curdy light green copper salt, very sparingly soluble in water, was at once precipitated. It was dried at 110° and analysed as follows:

0·1036 g.; 0·0201 g. CuO
0·1027 g.; 0·0193 g. CuO
0·2098 g. neutralised 5·0 cc. *N*/10 acid (Kjeldahl).

	Cu	N
Found	15·4, 14·9	3·34
Calculated for $C_{15}H_{13}O_7NSCu$	15·3	3·37

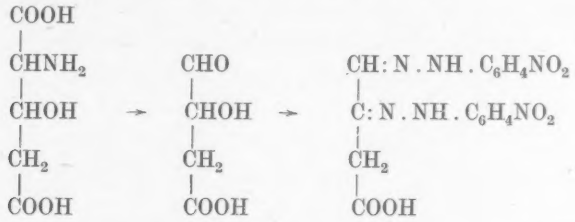
Oxidation of the acid with Chloramine-T. A normal solution (5 cc.) of the acid, containing 0·815 g., was neutralised with normal sodium hydroxide (5 cc.) and then mixed with 1·44 g. chloramine-T (sodio-*p*-toluenesulphochloroamide), dissolved in 5 cc. of water. An immediate reaction took place with precipitation of toluenesulphonamide, but active chlorine did not disappear until after about an hour. The solution was filtered and then warmed on the water-bath with a solution of *p*-nitrophenylhydrazine (2 g.) in dilute alcoholic sulphuric acid. On heating, it appeared that the hydrazone first formed was being converted into a sparingly soluble osazone. After three-quarters of an hour, the precipitate was filtered off with suction and well washed with alcohol. The washed product, weighing about 0·6 g., was recrystallised from boiling nitrobenzene, and separated out as red-brown prismatic needles melting at 297–299°. The substance is sparingly soluble in most neutral solvents but is very freely soluble in pyridine giving a yellow crystalline salt. Alcoholic ammonia gives a red crystalline salt. On adding sodium hydroxide to an alcoholic solution containing a trace of the substance, a deep blue colour is produced. This reaction is apparently characteristic of two adjacent nitrophenylhydrazine groups.

For analysis, the substance was dried *in vacuo* at 120° over phosphorus pentoxide.

0·1142 g.; 0·2140 g. CO_2; 0·0397 g. H_2O
0·1250 g.; 0·2353 g. CO_2; 0·0432 g. H_2O
0·1650 g.; 34·0 cc. N at 26° and 756 mm. (Dumas).

	C	H	N
Found	51·1, 51·3	3·86, 3·84	22·7
Calculated for $C_{16}H_{14}O_6N_6$	51·1	3·72	22·6

Assuming the acid to be correctly represented as a β-hydroxyglutamic acid, the course of the reaction with chloramine-T[1] and the subsequent osazone formation would almost surely be represented as follows:



No attempt to isolate the free aldehyde has yet been made, but this will be tried later, as well as its conversion into malic acid.

Reduction of the Acid with Hydriodic Acid. The reduction of hydroxyglutamic acid with hydriodic acid presents some difficulties. In all the experiments, one part of the acid (0·5–1 g.) was heated with 0·3 part red phosphorus and 10 parts of fuming hydriodic acid (sp. gr. 1·9). At a low temperature, such as 120° on boiling under a reflux condenser, little or no change occurs, whilst in a sealed tube at high temperatures (180–200°) any glutamic acid formed is decomposed with formation of butyric and other products [cf. Kwisda, 1891]. On the other hand for the reduction of β-hydroxyglutaric acid by hydriodic acid, a temperature of 180° is desirable, according to Pechmann and Jenisch [1891]. The best result was obtained by heating the mixture in a sealed tube at 145–150° for six hours. The clear contents of the tube were diluted with water (100 cc.) and boiled with excess of precipitated lead hydroxide until neutral. After filtering, the lead was removed from the filtrate with hydrogen sulphide and the solution concentrated to a syrup. Fuming hydrochloric was then added and the mixture allowed to stand in the ice box. Characteristic crystals of glutamic acid hydrochloride soon separated and they were filtered off and recrystallised again from hydrochloric acid. The crystals melted at 195–197° and a mixed melting point determination with *d*-glutamic acid hydrochloride of other origin melted at the same temperature. The yield of pure product in two experiments was only between 10 and 15 per cent. The substance gave the pyrrole reaction strongly. It was analysed for nitrogen by van Slyke's method:

0·0132 g.; 1·75 cc. N at 21° and 765 mm. = 7·57 per cent. N.
Calculated for $C_5H_9O_4N$ HCl = 7·65 per cent. N.

[1] For analogous oxidations of aspartic and glutamic acids with chloramine-T see Dakin [1917].

Colour Reactions of the Acid. On adding excess of concentrated sulphuric acid to a few drops of a dilute solution of the acid together with a few milligrams of a phenol, a variety of rather definite colour reactions are obtained some of which may be of use for purposes of identification. Perhaps the reactions with resorcinol and α-naphthol are the most striking and sensitive. It should be noted that the absence of any trace of nitrates is essential or misleading results will be obtained.

Resorcinol. Clear reddish purple turning brown on warming.

Catechol. Blue green.

Thymol. Clear bright green.

Pyrogallol. Dark green.

Phloroglucinol. Cherry red.

α-Naphthol. Clear fluorescent green.

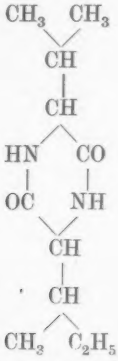
β-Naphthol. On warming, a yellowish green fluorescent solution resembling the similar reaction with malic acid.

The acid also appears to give a red colour reaction with diazobenzenesulphonic acid when warmed with excess of sodium hydroxide. This reaction due to Rosenthaler [1912] is given by various alcohols and especially by malic acid. Minute traces of histidine are apt to complicate the reaction.

III. A new Peptide, Isoleucylvaline anhydride, obtained by the acid hydrolysis of Caseinogen.

In the first of the preceding sections, an account was given of the separation of certain peptide anhydrides from the crude butyl alcohol extract from hydrolysed caseinogen. The peptide mixture is fairly easily soluble in butyl alcohol and was separated from proline by successive crystallisation from absolute alcohol and water—the proline remaining in solution. The two fractions, from alcohol and water respectively, were mixed together and averaged about 5 per cent. of the weight of caseinogen originally hydrolysed. The mixture is evidently composed of a number of different compounds and would repay careful examination. A tyrosine-containing peptide was separated by the action of cold normal sodium hydroxide and subsequent precipitation with acetic acid. Its complete identification still remains to be accomplished. The substance remaining insoluble in cold dilute alkali was recrystallised first of all from boiling absolute alcohol, in which it is sparingly soluble, and subsequently from 50 per cent. acetic acid, in which it dissolves readily. The crystalline product thus obtained was in the form of woolly

masses of fine needles, often over 1 cm. in length, and appeared perfectly uniform under the microscope. The yield was considerable, amounting in one case to slightly over 1 per cent. of pure product, so that the actual amount present must have been considerably more. The substance was almost insoluble in water and in dilute acid or alkali, even on moderate heating. In cold 50 per cent. acetic acid, its solubility was very close to 0·35 per cent., but much greater in the hot acid. It was sparingly soluble in alcohol, acetone or ethyl acetate, and insoluble in ether. It dissolved in concentrated sulphuric acid without developing colour. The substance did not react when boiled with copper oxide to give a copper salt, nor did it evolve nitrogen readily when treated with nitrous acid. These reactions clearly indicated the presence of the diketopiperazine type of linkage. At first the substance was thought to be leucinimide, but this was excluded by both melting point and analysis. The results of the analysis agreed with a *l*-leucyl-*d*-valine anhydride and such a substance has been isolated by Abderhalden and Funk [1907] from caseinogen and has been synthesised by Fischer and Scheibler [1908]. The melting points given for these products agree closely with one another, namely 285° (uncorr.) and 282° (corr.) respectively. On the other hand, the melting point of the substance under investigation was constant at 310–312° (uncorr.), so that there could be no question of identity, though possibly of stereoisomerism. But on hydrolysing the new substance with hydrobromic acid, good evidence was obtained of the presence of both *d*-isoleucine and *d*-valine, so that the original peptide was undoubtedly a *d*-isoleucyl-*d*-valine anhydride, *i.e.* isopropyl-*sec*-butyldiketopiperazine with the following structure:



Analysis. The substance was recrystallised from 50 per cent. acetic acid dried *in vacuo* at 110°.

0·1003 g.; 0·2281 g. CO_2; 0·0884 g. H_2O
0·1030 g.; 0·2343 g. CO_2; 0·0858 g. H_2O
0·1000 g. neutralised 9·45 cc. $N/10$ acid (Kjeldahl).

	C	H	N
Found	62·2, 62·0	9·79, 9·26	13·2
Calculated for $C_{11}H_{20}O_2N_2$	62·2	9·5	13·2

Optical Rotation. A 1 per cent. solution of the substance in glacial acetic acid was examined. Two different specimens gave the following values:

$c = 1·0$, $l = 2$ dm., $\alpha = -0·87°$ hence $[\alpha]_D^{20°} = -43·5°$,
$c = 1·0$, $l = 2$ dm., $\alpha = -0·84°$,, $[\alpha]_D^{20°} = -42·0°$.

Hydrolysis. One gram of the peptide was heated in a sealed tube with 10 cc. hydrobromic acid (sp. gr. 1·48) at 115–120° for eight hours. A little brown insoluble matter was filtered off and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in water and made just alkaline to ammonia and allowed to crystallise. The crystalline residue was washed with a little water and alcohol and dried at 90°. The mixed amino-acids were analysed for nitrogen and examined optically.

0·1351 g. neutralised 10·7 cc. $N/10$ acid (Kjeldahl) = 11·09 per cent. N.

0·1502 g. dissolved in 10 cc. of 20 per cent. hydrochloric acid, showed a dextro-rotation of + 0·96° in a 2 dm. tube, hence $[\alpha]_D = +32·1°$.

It will be seen that these figures agree closely with the mean of the values for *d*-valine and *d*-isoleucine. *d*-Valine contains 11·97 per cent. nitrogen and has a rotation of + 29·6°, while *d*-isoleucine contains 10·69 per cent. of nitrogen and has a rotation in hydrochloric acid of + 36·8°. The fact that the rotation of the mixed amino-acids from the peptide had as high a rotation as + 32·1°, is almost conclusive evidence of the presence of *d*-isoleucine and *d*-valine, as will be readily seen on consulting a table of optical rotation constants for the amino-acids.

The separation of *d*-isoleucine and *d*-valine is a difficult matter, especially when only small amounts of material are available. A partial separation of the residual amino-acid mixture was accomplished by taking out a fraction containing most of the *d*-isoleucine as a lead salt, according to Levene and van Slyke's method. The filtrate containing most of the valine was treated with hydrogen sulphide and recovered as described by the above authors. Since the optical properties of the two fractions were of greater importance than analytical figures, the rotations were observed first and after reading nitrogen estimations made on aliquot parts of the solution.

The crude *d*-isoleucine, 0·1200 g., dissolved in 10 cc. of 20 per cent. hydrochloric acid, showed a rotation of + 0·82° in a 2 dm. tube, hence $[\alpha]_D = + 34·2°$. It contained 10·90 per cent. of nitrogen. The crude *d*-valine contained 11·31 per cent. of nitrogen and 0·1500 g. in 10 cc. of 20 per cent. hydrochloric acid showed a dextro-rotation of + 0·92°, hence $[\alpha]_D = + 30·7°$. While the comparison of the above figures with the calculated shows that the separation of the isoleucine and valine was incomplete, it also appears to offer satisfactory evidence of the presence of both of them in about equal amounts.

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XXVII. OBSERVATIONS ON SOME METALLIC COMPOUNDS OF HAEMATOPORPHYRIN.

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Introduction.

It has long been recognised that haematoporphyrin forms at least three distinct types of compounds. It unites with mineral acids to form crystalline salts, *e.g.* the hydrochloride [Zaleski, 1902], in which the pigment plays the part of a base or cation, secondly it unites with strong bases to form salts, *e.g.* sodium, potassium and ammonium compounds [Nencki and Sieber, 1888], in which the pigment functions as an acid or anion, and thirdly it forms so-called metallic compounds, in which the metals enter into a more or less stable organic combination with the pigment, the metals ceasing to exist as ions. The absorption spectrum of haematoporphyrin in alkaline solution is probably to be regarded as that of the anion and the spectrum of the pigment in acid solution as that of the cation. An absorption spectrum of neutral haematoporphyrin has also been described, which may be that of the non-ionised pigment [Garrod, 1896]. The first two types are readily convertible into one another, while the pigments belonging to the metallic type are comparatively stable substances, their formation being in many cases an irreversible reaction.

The present paper deals mainly with the third class of compounds, being, in the first instance, an investigation of the spectroscopic characters of a number of synthetic compounds of pure haematoporphyrin, prepared by Nencki's method [Nencki and Zaleski, 1900], with various metallic hydroxides. The work of previous observers will be referred to in connection with each pigment.

A diffraction spectroscope was chosen for making the observations, since it yields a normal spectrum and the measurement of wave lengths is therefore greatly facilitated. On the other hand, there are certain disadvantages in the

use of a diffraction spectroscope as compared with a prismatic one for the observation of absorption spectra. The relatively great dispersion in the red region of the diffraction, as compared with the prismatic, spectrum results in absorption bands in that area being spread over a greater length with a consequent reduction in their intensity. The fact that the sensitivity of the eye for red rays is very low, the region of maximal sensitivity being about 530$\mu\mu$ [Ebert, 1888], further increases the difficulty of visual observation in this region. The question of the comparative efficiency of prismatic and diffraction spectra is fully discussed by Kayser [1900], from whom the following quotation is taken.

"Let us consider a prismatic spectrum, which has the same dispersion in the middle of the visible region as a diffraction spectrum, then it will be found that the prismatic spectrum while relatively extended in the blue and violet region is contracted in the red. Since it is just the latter parts which have a low intensity of light, observation in the prismatic spectrum is still possible, when it has ceased to be so in the diffraction spectrum."

Prism spectroscopes are therefore on the whole better adapted for the visual examination of pigments in gradually decreasing molecular concentrations, especially when the pigments show absorption bands in the red region. My original intention was to prepare "absorption curves" or "curves of molecular vibrations" according to Hartley's method [Kayser, 1905; Baly, 1905, p. 410] with the aid of a prism spectroscope, but delay in obtaining suitable apparatus has compelled me to limit the present observations to solutions of only one or two molar concentrations. Although the observations are therefore incomplete, yet they have the advantage over previous analogous investigations of allowing one to compare similar molecular concentrations of the pigments. I hope shortly to be in a position to supplement the following results by plotting out the absorption curves of the pigments with the aid of a prism spectroscope provided with means for the rapid and accurate determination of wave lengths.

The instrument used for the following work was a spectrometer provided with a graduated circle reading by opposite verniers to one minute of arc, and a transparent diffraction grating containing 14,500 lines to the inch.

The source of light employed was a small carbon arc lamp, the light being focussed on the slit of the spectroscope. The plane of the grating was adjusted so as to be perpendicular to the incident light.

The wave lengths (λ) of the margins of the absorption bands are stated in $\mu\mu$ or 10^{-7} cm.

Two stock solutions of haematoporphyrin of $M/1000$ concentration were prepared, one an acid and the other an alkaline solution. In calculating the molar concentrations Willstätter's [Willstätter and Stoll, 1913, pp. 44, 406] formula, $C_{33}H_{38}N_4O_6$; M.W.= 586·349, was adopted as a basis.

0·0586 g. of pure haematoporphyrin was dissolved in glacial acetic acid, 1 g. of pure sodium acetate was added, and the solution was made up to 100 cc. with glacial acetic acid (Solution 1). An alkaline solution of the same concentration was made up by dissolving 0·0586 g. of haematoporphyrin in 85 per cent. alcohol containing 10 cc. of $N/10$ caustic potash, the solution being made up to 100 cc. in a standard flask with 85 per cent. alcohol (Solution 2).

The absorption spectra of haematoporphyrin in alkaline and in mineral acid solutions are first recorded for the sake of comparison with their metallic derivatives.

$M/10{,}000$ haematoporphyrin in alkaline solution prepared by diluting Solution 2 with 85 per cent. alcohol.

Thickness of layer	Band I	Band II	Band III	Band IV
25 mm.	624·5–615·4	575–561	540·3–527·8	508·9–491·4

10 cc. of Solution 1 were made up to 100 cc. with 85 per cent. alcohol containing sulphuric acid (2 cc. of the concentrated acid in 100 cc. alcohol). The resulting solution is therefore $M/10{,}000$, and shows the following absorption bands.

Thickness of layer	Band I	Band II	
25 mm.	600·7–589·3	577·3–560·5	540·3
		Faint absorption	Dark absorption

I. *The conditions under which the metallic compounds are formed.*

Haematoporphyrin was found to combine with certain metals in the presence of excess of alkali or in acetic acid solution. When the metallic hydroxide was soluble in ammonia, that alkali was used. On the other hand, it is necessary to employ caustic soda or caustic potash as the alkali in the preparation of the lead compound. No combination takes place in the presence of free mineral acids. When salts of the metals with mineral acids are added to solutions of haematoporphyrin in glacial acetic acid, it is therefore necessary to add at least an equivalent amount of sodium acetate.

II. *Compounds of haematoporphyrin with certain metals.*

1. *Zinc and Cadmium compounds.*

The absorption spectrum of the zinc compound was first described by MacMunn [1889], later by Garrod [1892], and Hammarsten [1893]. It was prepared by the action of zinc chloride on an ammoniacal solution of haematoporphyrin. A $M/5000$ solution of the zinc compound in ammoniacal alcohol shows the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	591·2–569·2	559·1–527·3
10 mm.	587·4–572·1	551–534

The zinc compound can also be easily prepared as a $M/5000$ solution in glacial acetic acid in the following way. 0·01 g. of zinc acetate is added to 10 cc. of $M/1000$ solution of haematoporphyrin in glacial acetic acid. The solution is then heated for about 20 minutes on the water-bath, cooled, and made up to 50 cc. with glacial acetic acid.

The following was its absorption spectrum:

Thickness of layer	Band I	Band II
10 mm.	585–568	550–532

A $M/5000$ solution of the cadmium compound was prepared in the following way. 10 cc. of the $M/1000$ alkaline solution of haematoporphyrin were mixed with 5 cc. of concentrated ammonia, and 0·2 cc. of $M/1$ cadmium chloride, and the solution made up to 50 cc. with 50 per cent. alcohol. After standing at room temperature for 24 hours, the solution showed the following absorption bands on spectroscopic examination.

Thickness of layer	Band I	Band II
25 mm.	596·4–578·8	560·5–542·74
10 mm.	Too faint to define	556·2–543·2

The cadmium compound cannot be prepared in solution in glacial acetic acid. Both the zinc and cadmium compounds are unstable substances, being at once converted into acid haematoporphyrin on acidification with hydrochloric or sulphuric acid.

2. *Compounds of haematoporphyrin with nickel, cobalt and iron.*

These compounds are most readily prepared from solutions of haematoporphyrin in glacial acetic acid, but can also be prepared from ammoniacal solutions. The latter two compounds were first prepared by Laidlaw [1904], and the nickel compound by Zaleski [1904].

A $M/5000$ solution of the nickel compound was prepared in the following way. 5 cc. of a $M/1000$ solution of haematoporphyrin in glacial acetic acid

were mixed with 5 cc. of *N* sodium acetate dissolved in glacial acetic acid and 0·005 g. of nickel acetate. The solution was then heated on the water-bath for about half-an-hour, allowed to cool and made up to 25 cc. with glacial acetic acid. The cobalt compound was prepared in a similar way, 0·01 g. of cobaltous chloride being added instead of the nickel salt.

These solutions showed the following absorption bands on spectroscopic examination.

M/5000 Nickel Compound.

Thickness of layer	Band I	Band II
10 mm.	558·15–543·7	(522·9–510)?

M/5000 Cobalt Compound.

25 mm.	567·8–552·9	539·4–515·2
10 mm.	564·9–553·3	(531·15–516·15)?

In both cases the first bands are well defined and persist on considerable dilution. The second bands are much fainter and their margins are difficult to define with the diffraction spectroscope. The absorption spectra of both pigments bear a striking resemblance to that of haemochromogen. The addition of mineral acid to solutions of these compounds does not produce any distinct change in their absorption spectra. They differ, therefore, from the zinc and cadmium compounds in being stable in the presence of the mineral acid. The following experiments prove this statement: 25 cc. of *M*/5000 solutions of the pigments were mixed with an equal volume of 85 per cent. alcohol strongly acidified with sulphuric acid (10 cc. of the concentrated acid made up to 100 cc. with 85 per cent. alcohol). The resulting solutions were therefore *M*/10,000.

Nickel Compound.

Thickness of layer	Band I	Band II
25 mm.	560·55–542·7	523·9–507·4?

Cobalt Compound.

25 mm.	567·3–553·3	533·6–519·2?

The synthetic compound of haematoporphyrin with iron is somewhat more difficult to prepare. The following method was found to lead to the desired result: 0·03 cc. of *M*/2 ferric chloride solution was placed in a 25 cc. flask, and 10 cc. of glacial acetic acid and a trace of pure iron wire were added to convert the ferric into a ferrous salt. The addition of metallic iron is essential. No interaction was found to take place between haematoporphyrin and iron when present as a ferric salt. On the other hand Willstätter and Stoll [1913, p. 412] have proved that iron in the ferric form can be linked with aetio-porphyrin in the presence of an equivalent amount of sodium acetate. The

solution was then heated to the boiling point on the oil-bath for some minutes, 5 cc. of *M*/1000 haematoporphyrin dissolved in glacial acetic acid were added and the fluid was maintained nearly at the boiling point for fully an hour. Finally the solution was cooled and made up to 25 cc. with glacial acetic acid. An absorption band in the red region of the spectrum closely resembling in position and character the corresponding band of acid haematin can easily be distinguished on examination with a direct vision spectroscope. This band is somewhat difficult to define on examination with the diffraction spectroscope.

M/5000 Synthetic Haematin in acid Solution.

Thickness of layer	Band I
25 mm.	633–611·69

A *M*/5000 solution of pure haematin (M.W. 621·14 Willstätter), prepared from crystalline haemin, in glacial acetic acid showed the following absorption band in the red region on examination with the diffraction spectroscope. A layer of 45 mm. was found to be necessary in order to define the limits of the band with the diffraction spectroscope.

Thickness of layer	Band I
45 mm.	647·2–612

Although the fact, that the thicknesses of the layers examined were different for the two pigments, renders comparison difficult, it will be noticed that the absorption band lies farther towards the extreme red end of the spectrum in the case of natural haematin than in that of the synthetic pigment.

Since the absorption bands of reduced haematin in alkaline solution are better defined and more persistent than those of acid haematin, it was considered necessary to compare the reduced forms of the synthetic and natural pigments in alkaline solution.

Synthetic reduced haematin or haemochromogen in alkaline solution was therefore prepared in the following way from haematoporphyrin. 5 cc. of *M*/1000 haematoporphyrin dissolved in glacial acetic acid, 0·03 cc. of *M*/2 solution of ferric chloride and a trace of iron wire were heated in the same way as the previous preparation. The solution was cooled and rendered alkaline by the very gradual addition of concentrated ammonia, the solution being cooled from time to time by immersion in cold water. 0·3 g. of hydrazine hydrochloride was then added and the solution was made up to 25 cc. with 50 per cent. ethyl alcohol. This *M*/5000 solution of reduced synthetic haematin in alkaline solution or haemochromogen showed the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	562·47–556·2–539·4	526·8–506·9
	Faint absorption Dark absorption	
10 mm.	552·9–543·2	524·4–512·8

Band II is much less intense and well defined than Band I.

For comparison with the synthetic pigment, a $M/5000$ solution of haemochromogen was prepared from pure haematin in the following way: 0·1553 g. of pure haematin (M.W. = 621·14) [Willstätter and Stoll, 1913, p. 35] was placed in a 100 cc. flask, dissolved in 70 per cent. ethyl alcohol containing 2 cc. of N caustic soda, and the solution was made up to 100 cc. with 70 per cent. alcohol. The concentration of this solution was therefore $M/400$. 4 cc. of this solution were placed in a 50 cc. flask, 5 cc. of N caustic potash and 0·5 g. of hydrazine hydrochloride were added. Finally the solution was made up to 50 cc. with N ammonia. This $M/5000$ solution of haemochromogen showed the following absorption bands on spectroscopic examination.

Thickness of layer	Band I	Band II
25 mm.	566·3–542·3	529·7–508·4
10 mm.	557·7–545·15	527·3–513·7

The absorption spectra of natural and synthetic haematin are therefore similar, but not identical.

The similarity of their absorption spectra indicates that the nickel and cobalt compounds are closely related to the reduced form of haematin. They differ from haemochromogen in their stability, being resistant to the action of mineral acids and unaffected by exposure to air. Haemochromogen, on the other hand, is rapidly oxidised to haematin on exposure to air. The stability of the nickel and cobalt compounds is probably due to the fact that both metals form stable divalent compounds; while divalent iron tends to undergo conversion into the trivalent form. Haemochromogen prepared from haematin is so unstable in acid solution, that special precautions have to be taken in its preparation [Milroy, 1905; Dhéré, 1917].

3. *Compound of haematoporphyrin with copper.*

This substance was first prepared and described by Laidlaw [1904], who also proved that it is identical with or very closely allied to the pigment turacin [Church, 1869, 1892]. I think that the pigment prepared from the urine and described by Saillet [1896] as "iron free haemochromogen" was probably also this copper compound. Saillet obtained it by the action of ammonia on urinary haematoporphyrin. Recent work [C. Powell White] indicates that a minute trace of copper is present as a constant constituent

of urine, and it, therefore, seems probable that the pigment was formed by the action of a trace of a copper salt on the ammoniacal solution of haematoporphyrin. I have repeated Saillet's experiment using pure haematoporphyrin without obtaining the "iron free haemochromogen."

The cupric compound can be easily prepared either in ammoniacal solution, or dissolved in glacial acetic acid. A $M/5000$ solution in glacial acetic acid was prepared in the following way. 0·002 g. of cupric acetate was added to 5 cc. of $M/1000$ haematoporphyrin dissolved in glacial acetic acid, 15 cc. of $N/10$ sodium acetate dissolved in glacial acetic acid were added, and the solution heated on the water-bath for about 20 minutes. The solution was then allowed to cool, and made up to 25 cc. with glacial acetic acid. The following absorption spectrum was found on examination.

Thickness of layer	Band I	Band II
25 mm.	587·8–580·2–553·3	539·4–516·6
	Faint absorption / Intense absorption	
10 mm.	571–557·7	535–519·5

A $M/10{,}000$ solution of the copper compound in alcohol containing sulphuric acid of the same concentration as that used in the preparation of the similar solution of the nickel compound gave the following absorption spectrum.

Thickness of layer	Band I	Band II
25 mm.	572·5–553·3	538·4–517·12

Band II is much less intense and persistent than Band I.

The copper compound therefore resembles the nickel and cobalt derivatives in being stable in the presence of free mineral acids.

4. *Compound of haematoporphyrin with tin.*

Haematoporphyrin also forms a compound with tin which is characterised by two well-defined and persistent absorption bands, and by its great stability in mineral acid solutions.

A $M/5000$ solution of this pigment was prepared in the following way. 0·5 cc. of M stannous chloride was added to 5 cc. of $M/1000$ haematoporphyrin dissolved in glacial acetic acid, 8 cc. of N sodium acetate dissolved in glacial acetic acid were then added, the solution was heated for 20 minutes on the water-bath, then cooled, and made up to 25 cc. with glacial acetic acid. The resulting solution was filtered, and examined spectroscopically.

Thickness of layer	Band I	Band II
10 mm.	580·7–568·7	547·5–526·8
5 mm.	579·25–569·2	545·6–528·7

A M/10,000 solution of the stannous compound in alcohol containing sulphuric acid was prepared in the same way as the corresponding solutions of the other pigments and showed the following absorption spectrum.

Thickness of layer	Band I	Band II
25 mm.	580·7–568·2	547·5–525·4

The stannous compound can not only be prepared synthetically from haematoporphyrin, but also directly from haematin, or from defibrinated blood.

The following method was used for preparing the pigment from pure haematin. 4 cc. of a M/400 solution of pure haematin, prepared in the way previously described, were placed in a 50 cc. flask, 20 cc. of glacial acetic acid were added, and the solution was then heated on the water-bath for 20 minutes. 3 cc. of 2M stannous chloride were then added and the heating continued for one hour. On examining this solution with a small direct vision spectroscope, the absorption spectrum of acid haematoporphyrin was recognised. 15 cc. of N sodium acetate in glacial acetic acid were added, and the fluid was again heated for half an hour. The cooled solution was then made up to 50 cc. with glacial acetic acid. One portion of the solution was diluted with an equal volume of ethyl alcohol so as to form a M/10,000 solution. Another portion was diluted with an equal volume of alcohol containing sulphuric acid of the same concentration as that used in the previous preparations. The filtered solutions showed the following absorption spectra.

Nature of solution	Thickness of layer	Band I	Band II
M/10,000 Sn compound in acetic acid and alcohol	25 mm.	586–569·7	548–531·6
M/10,000 Sn compound in sulphuric acid and alcohol	25 mm.	587–570	552–530

It will be noticed that the absorption bands are situated nearer the red end of the spectrum than those of the synthetic pigment, and that no marked change is produced in the absorption spectrum by the action of sulphuric acid.

The absorption bands of the stannous compound are very persistent. A M/250,000 solution of the synthetic pigment still shows faintly the two characteristic absorption bands examined in a layer of 10 mm. with a direct vision spectroscope. Its absorption bands have a greater power of resisting dilution than those of haemochromogen. The more intense of the two

absorption bands of a $M/100{,}000$ solution of pure haemochromogen, prepared by the reduction of haematin in alkaline solution with hydrazine hydrate could not be detected in a layer of 10 mm.

Stannous compound as a test for blood pigment.

The formation of the stannous compound can therefore be utilised as a very delicate test for blood pigment. The test may be rapidly carried out in the following way. Three drops of defibrinated ox blood are added to 5 cc. of glacial acetic acid placed in a test tube. The test may also be carried out equally well with much less blood, *e.g.* one drop of blood to 10 cc. of glacial acetic acid. The use of a larger amount of blood has the advantage of allowing one to trace clearly the intermediate stages in the formation of the stannous compound. The solution is then heated to the boiling point. It will be found to show the absorption spectrum of acid haematin. Three drops of $2M$ stannous chloride are added, and the solution is boiled for about one minute. When the precipitate, which forms, has been filtered off or allowed to settle, the absorption bands of acid haematoporphyrin can easily be seen on spectroscopic examination. A small quantity of solid sodium acetate is then added, and the solution again boiled. The fluid which has become brighter red in colour, is cooled and filtered. On spectroscopic examination of the filtrate the two characteristic absorption bands of the stannous derivative will be clearly seen. They closely resemble in position and character the bands of oxyhaemoglobin.

The application of the method to the examination of suspected stains for dried blood is very simple. A portion of the stained tissue is extracted by boiling with a little glacial acetic acid in a small test tube, and the solution of haematin thus obtained is treated in the way already described.

The following simple experiments illustrate the delicacy of the reaction as a test for blood pigment compared with the haemochromogen test.

1 cc. of diluted defibrinated ox blood (1 in 10 dilution in water) was added to 7 cc. of glacial acetic acid, boiled for a few minutes, mixed with four drops of $2M$ stannous chloride and again boiled gently for about five minutes. A small quantity of sodium acetate was added to the warm solution. which was then again boiled, allowed to cool, and finally made up to 10 cc. with glacial acetic acid; 5 cc. of this solution made up to 50 cc. with ethyl alcohol, and filtered, showed very distinctly the two absorption bands of the stannous derivative on spectroscopic examination. 5 cc. of the latter solution were diluted to 25 cc. and again filtered. The final stage of dilution therefore

corresponded to one volume of blood in 5000. When this solution was examined in a layer 25 mm. thick, the two absorption bands of the stannous compound could be seen, although they were faint.

The haemochromogen test was carried out for comparison in the following way.

10 cc. of a 1 in 1000 dilution of defibrinated ox blood were heated with 1 cc. of concentrated ammonia, then mixed with 5 cc. of ammonium sulphide solution, and made up to 20 cc. with water. Spectroscopic examination of this solution which corresponds with one volume of blood in 2000, showed one very faint absorption band. At a dilution of 1 in 5000, no absorption band could be detected in a layer of 25 mm.

A method for increasing the delicacy of the test.

After several other methods had been tried, the following method was found to give the most satisfactory results. Since known molecular concentrations of the various pigments have been dealt with throughout the greater part of this paper, it was considered advisable to work with solutions of oxyhaemoglobin of approximately known molecular concentration. Defibrinated ox blood having a specific gravity of 1050 was used for the test. Abderhalden's [1898] determinations of the iron as ferric oxide in ox blood were taken as a basis for calculation.

100 g. of ox blood contain 0·03805 g. of iron (calculated from Abderhalden's figures for ferric oxide). The ox blood used had a specific gravity of 1050 and 100 cc. of it therefore contained 0·03995 g. of iron; 139·8 cc. of ox blood would consequently contain 0·05584 g. of iron, and would be equivalent to 100 cc. of a $M/100$ solution of iron. 60·2 cc. of water were measured into a 200 cc. flask, and the solution was made up to 200 cc. with blood. The resulting solution is $M/200$ as regards its iron content, and, on the assumption that all the iron is present in combination as haemoglobin, the solution would also be approximately a $M/200$ solution of oxyhaemoglobin. 10 cc. of this solution were made up to 250 cc. with water. The resulting solution of oxyhaemoglobin was therefore $M/5000$. It may be, incidentally, of some interest to give the results of a spectroscopic examination of this solution compared with one of carbonic oxide haemoglobin of the same concentration.

Pigment	Thickness of layer	Band I	Band II
HbO_2	10 mm.	585 –567	550·7–527
HbCO	10 mm.	582·1–560	548·5–522·9

2·5 cc. of the $M/5000$ solution of oxyhaemoglobin were transferred to a 500 cc. flask, and made up to 500 cc. with water. The resulting solution of

oxyhaemoglobin was therefore $M \times 10^{-6}$. 150 cc. of this solution were measured into a 250 cc. flask, 6·3 g. of pure re-distilled phenol were added, the fluid was gradually heated on the water-bath, and then kept at about 90° for 20 minutes. The solution was then saturated with ammonium sulphate (about 114 g.). Sodium chloride or sodium sulphate may be used instead of ammonium sulphate, but are less effective, the separation of the phenol being slower and apparently less complete, especially in the case of sodium chloride.

The phenol, after being thus "salted out," rose to the surface of the saline solution as a brown coloured layer containing the blood pigment in the form of haematin. Sufficient saturated solution of ammonium sulphate was added to the cooled fluid to bring the phenol solution entirely into the narrow neck of the flask. A small quantity of the phenol solution was transferred by means of a pipette to a test tube, filtered, mixed with half its volume of glacial acetic acid, and heated nearly to the boiling point. 4–6 drops of $2M$ stannous chloride were added and the solution was boiled for about two minutes, a few crystals of sodium acetate were added, and the solution was again boiled, and filtered. On examining the filtrate, placed in a test tube of about 11 mm. diameter, with the aid of a small direct vision spectroscope, the two characteristic absorption bands of the stannous compound were distinctly visible.

It is possible by means of this method to detect the presence of blood pigment in a concentration of $10^{-6} \times M/5$. In the latter case, 200 cc. of a $10^{-6} \times M/5$ solution of oxyhaemoglobin were placed in a 300 cc. flask, and 5·9 g. of phenol were added. The rest of the method was carried out in the same way as for the solution of higher concentration.

I believe that the foregoing test will be found to be the most delicate specific reaction for blood pigment as yet available, the guaiac, benzidine, phenolphthalin, and similar tests not yielding absolutely conclusive results.

Isolation of the stannous compound.

Attempts to isolate the stannous compound in a pure state have not been entirely successful. The following is a description of the method used in the preparation of the pigment. 10 g. of stannous chloride, 10 cc. of concentrated hydrochloric acid, and about 2 g. of pure finely granulated tin were heated with 100 cc. of glacial acetic acid in a round bottomed flask of one litre capacity until the greater part of the tin had dissolved. 400 cc. of glacial acetic acid and 25 g. of anhydrous sodium acetate were then added. While this solution was kept at 90°, 100 cc. of defibrinated ox blood were gradually

added. The flask containing the solution was then closed with a rubber stopper provided with a glass tube and stopcock. The solution was allowed to cool to about 80°, the flask was evacuated, care being taken that none of the fluid was lost by frothing, the glass tap was turned so as to cut off the interior of the flask, and the flask together with the glass tap was detached from the aspirating pump. The object of heating the fluid *in vacuo* was to hinder re-oxidation of the reduced haematin by the oxygen of the air. The evacuated flask was then heated on the water-bath to about 80° for 45 minutes. The solution acquired a bright red colour. A small sample of the fluid after dilution with glacial acetic acid showed on spectroscopic examination the two characteristic absorption bands of the stannous compound.

On standing for 24 hours, a large proportion of the pigment was found to have separated out mainly in the amorphous form, but partly as small rhombic crystals of red colour. At first, it was hoped that the pigment might be separated from the mother liquid as in Schalfejeff's [1885] method for the preparation of haemin; but this proved to be impossible as the pigment settles very slowly from the solution. It also proved to be impossible to separate the pigment by means of the centrifuge. The jelly-like products of the action of the acetic acid on the proteins of the blood also render filtration impossible.

The following method was therefore adopted for freeing the pigment from the excess of stannous chloride, and from part of the proteins of the blood. The solution and suspension of the pigment in glacial acetic acid was concentrated nearly to dryness by distilling off the glacial acetic acid *in vacuo* on the water-bath. The residue was washed several times with water, saturated with potassium carbonate and extracted with ethyl alcohol. The alkaline alcoholic solution containing the greater part of the pigment was separated from the aqueous saline layer, filtered, and transferred to a distillation flask. The alcohol was then distilled off under reduced pressure. The pigment residue was dissolved in water, sufficient alkali being present to bring the greater part of it into solution, the liquid was then filtered, and the pigment was precipitated from the filtrate by the addition of dilute acetic acid. This precipitate was filtered off, washed with water, and dissolved in dilute sodium carbonate solution. The alkaline solution was then filtered, and the pigment again precipitated from the filtrate by the addition of dilute acetic acid. This precipitate was finally collected on filter paper, washed with water and dried *in vacuo* over sulphuric acid.

The pigment obtained in this way is insoluble in neutral acetone, ether,

ethyl and amyl alcohols and chloroform. It dissolves in ether, ethyl and amyl alcohols, and chloroform acidified with acetic acid; but only dilute solutions can be thus obtained. It is practically insoluble in acetone containing about 2 per cent. of acetic acid; but dissolves slightly in acetone acidified with hydrochloric or trichloracetic acid. It is only slightly soluble in pyridine, and in mixtures of pyridine with ethyl alcohol, but is more readily soluble in ammoniacal alcohol. It is practically insoluble in aniline; but dissolves readily in phenol. The pigment is insoluble in dilute aqueous mineral and organic acids, but is readily soluble in dilute aqueous solutions of the fixed caustic alkalies, in ammonia, and in dilute sodium or potassium carbonate solutions.

Further experiments are being made with the object of improving the method of preparation; but are at present too incomplete to justify description.

Determinations of the ash yielded by the pigment after purification by separation from saturated solutions in acid acetone and ammoniacal alcohol have not yielded constant percentages. The quantities of pigment dealt with were small varying from 0·14 up to 0·2972 g., and little stress can therefore be laid on the results. The solubility of the pigment in the above organic solvents is so slight that relatively large quantities of the solvents must be used in the preparation of small amounts of the pigment. One result of these estimations may prove of some significance, namely the fact that the percentage of stannic oxide yielded by the pigment was never higher than half that, which one would anticipate on the hypothesis that in the formation of the pigment one atom of tin replaces one atom of iron in the haematin molecule.

The stannous pigment is so resistant to the action of mineral acids that it may be dissolved in concentrated sulphuric acid without undergoing any distinct change in its absorption spectrum. The results of spectroscopic examination of solutions of the pigment of unknown strength in concentrated sulphuric acid are given in the following columns:

Pigment dissolved in H_2SO_4	Thickness of layer	Band I	Band II
Solution (1)	20 mm.	592·1–562·4	551·4–519
Solution (2) (more dilute)	20 mm.	581·1–568·7	542·7–527·8

This pigment is therefore even more resistant to the action of mineral acids than haematin.

5. *Compound of haematoporphyrin with lead.*

This compound is only formed in alkaline solution, being decomposed by acetic as well as by mineral acids. It was prepared in the following way.

10 cc. of $M/1000$ alkaline solution of haematoporphyrin, placed in a 50 cc. flask, were mixed with 25 cc. $N/10$ caustic soda and 0·3 cc. of $N/2$ solution of lead acetate. The solution was then heated for a few minutes to the boiling point, cooled and made up to 50 cc. with $N/10$ caustic soda. The following is the absorption spectrum of the compound:

Thickness of layer	Band
10 mm.	600–578
25 mm.	609–570

The solution has a greenish brown colour, and shows only one band in the visible spectrum.

The above mentioned metals are the only ones which have been found to form compounds with haematoporphyrin with the exception of manganese [Zaleski, 1904]. No spectroscopic evidence was obtained of the formation of metallic compounds with aluminium, uranium, silver, or magnesium in either acetic acid or alkaline solutions. The pigment, which I described in an earlier paper as resulting from the reduction of haematin with aluminium in acetic acid solution, owed its formation to the presence of a trace of tin in the aluminium powder [1901]. It is not formed when pure aluminium is used.

A SIMPLE METHOD FOR PREPARING METALLIC COMPOUNDS OF HAEMATOPORPHYRIN.

It is necessary to use pure haematoporphyrin for the preparation of metallic derivatives of known concentration; but, as a considerable amount of labour is involved in their preparation, the following simple method has proved of some value in facilitating the preparation of these pigments, and thus rendering them more accessible for observation. The proteins of defibrinated blood were precipitated with alcohol, dried and finely powdered. The precipitate, while still moist with alcohol, may also be used. 10 g. or somewhat less of the powder were then extracted with about 100 cc. of 85 per cent. ethyl alcohol acidified with sulphuric acid (2 cc. of the concentrated acid in each 100 cc. of alcohol). The dark brown solution of haematin thus obtained was filtered, a trace of aluminium powder was added to the filtrate, and the fluid was heated to the boiling point on the water-bath for at most three minutes. The solution, which acquired a bright red colour, was then filtered. Aluminium was selected as a reducing agent instead of zinc, since it does not form a compound with haematoporphyrin. On more prolonged heating the haematoporphyrin undergoes reduction, the solution becomes brown in colour and shows an absorption band in a similar position to that

of urobilin. It is therefore necessary to avoid more prolonged heating. A small portion of the filtrate was diluted with ethyl alcohol, filtered, and examined spectroscopically. It gave the absorption spectrum of haematoporphyrin in acid solution.

The greater part of the filtrate was then divided into two portions.

One of these was rendered alkaline with ammonia, and the precipitate of aluminium hydroxide, which formed, was filtered off. The alkaline filtrate, which on spectroscopic examination showed the bands of haematoporphyrin in alkaline solution, was divided into three portions. To one of these a trace of a copper salt, to another a zinc salt, and to a third a cadmium salt was added. The solutions were then heated on the water-bath for a few minutes, allowed to cool, diluted with alcohol, filtered, and examined with a spectroscope. They show the absorption spectra of the metallic compounds. The copper compound is formed very rapidly, the zinc and cadmium compounds more slowly. In the case of the latter two compounds it is advisable to allow their solutions to stand for some hours before diluting them with alcohol and examining them with the spectroscope.

Excess of finely powdered anhydrous potassium carbonate was gradually added to the other half of the acid solution. After each addition of potassium carbonate the evolution of carbon dioxide should be allowed to cease before more of the alkali is added. The fluid was then heated on the water-bath until the alcoholic solution became brown and reacted alkaline to litmus paper. After the alcoholic solution had been allowed to cool, it was separated from the aqueous saline layer, and filtered. A small portion after dilution with alcohol was found to give the absorption spectrum of alkaline haematoporphyrin.

Preparation of the lead compound. A considerable excess of *N* caustic soda was added to a small portion of the alcoholic solution, a few drops of a solution of lead hydroxide in caustic alkali were added, and the fluid was heated on the water-bath for a few minutes. The solution acquired a greenish tint and showed the absorption spectrum of the lead compound.

Preparation of the other metallic compounds. The remainder of the alkaline alcoholic solution was rendered acid by the addition of about one-fourth its volume of glacial acetic acid. Traces of zinc, copper and nickel acetates, cobaltous chloride, and stannous chloride were added to five separate portions of the acid solution. When chlorides or sulphates of the metals are added it is advisable also to add a little sodium acetate. The solutions were then heated on the water-bath for about 20 minutes, diluted with ethyl alcohol

or glacial acetic acid, filtered, and examined with the spectroscope. They showed the absorption spectra of the corresponding metallic derivatives.

Preparation of synthetic haematin in acid solution. A portion of the above mentioned acid solution was further diluted with twice or thrice its volume of glacial acetic acid, a trace of ferric chloride and a trace of iron wire were added, the solution was heated to the boiling point and kept in the water-bath for at least 30 minutes. On spectroscopic examination it was found to show the absorption spectrum of synthetic haematin in acid solution.

Comparative resistance to the effect of dilution shown by the absorption bands of the pigments.

Since the observations already made with the aid of the diffraction spectroscope only refer to solutions of one or two molar concentrations, and therefore leave the question of the relative intensity of the absorption bands practically untouched, it was considered essential to supplement them by determining approximately the concentrations at which the various absorption bands are just visible.

For this purpose, solutions of known molecular concentration were examined in layers of gradually diminished thickness until the absorption bands ceased to be visible. The solutions investigated were placed in a Baly's [1905, p. 413] adjustable cell, and examined with the aid of a prism spectroscope. The pigments were examined in the same order as those already investigated by means of the diffraction spectroscope. The wave lengths of the limits of certain additional absorption bands which became manifest in the red region of the spectrum are also recorded. The absorption bands previously defined are numbered in the same way as in the earlier part of this paper.

A *M*/10,000 alkaline solution of haematoporphyrin examined in a layer 37 mm. thick shows two bands in the red region in addition to those already described, (1) having the limits 680–665 and (2) having the limits 650–640. Six absorption bands are therefore visible at this concentration. When a *M*/100,000 alkaline solution of haematoporphyrin is examined, each of these bands just remains visible, when the thickness of the layer traversed by the light is not reduced beyond the limits stated in mm. given in the following columns:

M/100,000 Alkaline Solution of haematoporphyrin.

Band (1)	Band (2)	Band 1	Band 2	Band 3	Band 4
120 mm.	55 mm.	37 mm.	22 mm.	15 mm.	15 mm.

The two most persistent absorption bands are therefore 3 and 4.

A *M*/100,000 solution of haematoporphyrin in alcohol acidified with sulphuric acid gave the following results:

Band 1	Band 2
13 mm.	8 mm.

Both bands of the pigment in acid solution are therefore more persistent than those of the pigment dissolved in alkali.

1. *Zinc and Cadmium compounds.* A *M*/100,000 solution of the zinc compound dissolved in alcohol acidified with acetic acid gave the following results:

Band 1	Band 2
11 mm.	11 mm.

The two bands are therefore equally persistent.

A *M*/5000 ammoniacal solution of the cadmium compound examined in a layer of 25 mm. shows two faint bands in the red region in addition to those already described. These bands have the following positions: (1) 645 and (2) 630–612. Their power of resisting dilution was not determined. When the solution was allowed to stand for some hours, bands (1) and (2) disappear. They were, therefore, apparently due to the presence of some unchanged haematoporphyrin.

A *M*/50,000 solution of the cadmium compound gave the following results:

Band 1	Band 2
32 mm.	30 mm.

2. *Nickel, Cobalt and Iron compounds.* *M*/100,000 solutions of the nickel and cobalt compounds gave the following results:

Pigment	Band 1	Band 2
Nickel compound	12 mm.	26 mm.
Cobalt compound	11 mm.	15 mm.

It will be noticed that in both cases bands 2 are less persistent than 1; but in the case of the cobalt compound they are of more nearly equal intensity.

The results for an alkaline solution of haemochromogen prepared from pure haematin by reduction with hydrazine hydrate are given below for comparison:

Pigment	Band 1	Band 2
M/100,000 haemochromogen	12 mm.	55 mm.

The difference in intensity of the two bands is more marked in this case than in those of the nickel and cobalt compounds.

Iron compounds. The characteristic absorption band in the red region given by solutions of haematin, both natural and synthetic, is much less

persistent than the absorption bands of the foregoing pigments. The solutions examined were $M/25{,}000$; but the results are stated for $M/100{,}000$ concentration to secure uniformity.

Pigment	Band
Synthetic haematin	132 mm.
Natural haematin	160 mm.

The absorption band in the red of natural haematin is therefore less persistent than that of synthetic haematin. Both pigments were dissolved in alcohol acidified with acetic acid, the acid concentration being the same in both cases.

3. *Copper compound.* A $M/100{,}000$ solution of the copper compound dissolved in alcohol acidified with acetic acid gave the following results:

Band 1	Band 2
6 mm.	22 mm.

It will be noticed than Band 1 is more persistent than the absorption bands of any of the pigments hitherto examined. The absorption spectrum resembles those of the nickel and cobalt compounds and of haemochromogen in the marked difference in relative intensity of the bands.

4. *Stannous compound.* A preliminary examination of a $M/100{,}000$ solution of the stannous compound of haematoporphyrin dissolved in alcohol acidified with acetic acid proved that further dilution was necessary. A $M/500{,}000$ solution was therefore examined, and gave the following results:

Band 1	Band 2
10 mm.	11 mm.

The two bands therefore appear to offer practically the same resistance to dilution. The absorption bands have also a greater intensity than those of any of the other metallic compounds of haematoporphyrin.

When higher concentrations of the stannous compound of haematoporphyrin are examined with a prism spectroscope, an additional band is seen in the red region of the spectrum.

The following is the absorption spectrum of a $M/5000$ solution examined in a layer of 25 mm.

Band (1)	Band 1	Band 2
638–620	598–563	560–517

When a $M/25{,}000$ solution of the stannous compound is examined in a layer 10 mm. thick, Band (1) can just be detected. It ceases to be visible when the thickness of layer is reduced beyond this amount.

The foregoing results are obviously incomplete not only as regards the limitations of the methods employed, to which reference has already been made in the introduction; but also because the observations are limited to the visible spectrum.

The great stability of the stannous compound gives rise to the view, that it would be interesting to find out what metabolic changes, if any, it undergoes within the animal body after subcutaneous or intravenous injection. The great persistence of the bands in its absorption spectrum would facilitate its recognition in the organism after injection. Further, in view of the marked optical sensitising action which haematoporphyrin exercises on the animal organism [Pfeiffer, 1911, and others] the question arises whether the stannous compound or other stable metallic derivatives of haematoporphyrin have a similar action.

At a later date, I hope to carry out work on this subject along these lines.

Summary.

1. The methods of preparation and the absorption spectra of the compounds of haematoporphyrin with zinc, cadmium, nickel, cobalt, iron, copper, tin and lead have been described.

2. The resistance of the pigments to the action of mineral acids has been determined.

3. A method for the direct preparation of the stannous compound from blood or haematin has been described.

4. A delicate test, based on the preparation of the stannous compound has been devised for the detection of traces of blood pigment.

5. A simple and rapid method for the preparation of metallic compounds of haematoporphyrin has been given.

6. The resistance of the absorption bands of the pigments to dilution has been approximately determined.

In conclusion, I should like to express my deep indebtedness to Professor W. B. Morton for lending me two spectrometers for use with grating and prism.

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XXVIII. THE PROTEINS OF COW'S COLOSTRUM. PART I. THE RELATION BETWEEN THE EUGLOBULIN AND PSEUDOGLOBULIN OF COW'S COLOSTRUM.

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In a previous communication [1915] the authors have brought forward data which demonstrate that the intramolecular arrangements of the amino-acids in the caseinogens of the cow and the sheep are not identical. This conclusion was arrived at from a comparison of the optical properties of the amino-acids derived from "racemised" cow's and sheep's caseinogens.

In subsequent work the method of protein racemisation has been extended successfully to the study of other proteins in an investigation which had for its object the elucidation of the relationships existing between the proteins of cow's colostrum and the corresponding compounds of ox-serum. In the present communication we give the results of the first part of this work, in which the relations between the globulins of cow's colostrum have been determined.

It has long been known that globulin, both in colostrum and in serum, consists of a mixture of two proteins, namely, euglobulin and pseudoglobulin. These compounds are characterised by widely different physical properties and solubilities. If a solution of globulin be submitted to dialysis, the euglobulin separates out, whilst the pseudoglobulin remains in solution.

The chemical relationship between the euglobulin and pseudoglobulin of serum has formed the subject of an investigation by Chick [1914], who concluded that the two proteins were very closely related. She found that pseudoglobulin preparations always contained, even at the end of a long dialysis, large quantities of a water-insoluble protein resembling euglobulin, and that the latter was largely derived from the pseudoglobulin by a gradual process of "denaturation." She suggested that this artificial euglobulin was

a mechanical complex originating from the interaction and mutual precipitation of two colloidal systems, viz. pseudoglobulin solution and lipoid emulsion. It appeared probable that serum euglobulin was a protein-lipoid complex of similar origin.

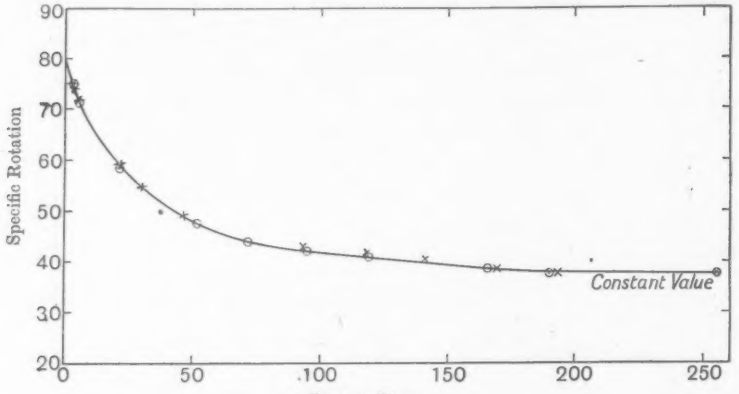
Further work by Hartley [1914] on this subject lends support to this view. The figures obtained by him in the analysis of serum euglobulin and pseudoglobulin by the method of van Slyke indicated that these substances were very similar in chemical composition.

Hitherto, little work of a comparative nature has been carried out with euglobulin and pseudoglobulin of cow's colostrum. In this paper we bring forward evidence in support of the view that the globulins of colostrum, just as of serum, are very closely related, if not actually identical, in chemical structure. Our experiments have indeed failed to reveal any structural differences at all between colostrum euglobulin and pseudoglobulin.

This conclusion has been arrived at by two independent methods of investigation, viz.

1. A comparison of the optical rotatory properties of euglobulin and pseudoglobulin in alkaline solution.

2. A comparative study of the optical properties of the amino-acids derived from the hydrolysis of "racemised" euglobulin and pseudoglobulin.



Graph showing progress of racemisation in $N/2$ alkali

If either of the globulins be dissolved in dilute alkali and the solution allowed to remain at 37° for several days, the initial rotation gradually diminishes until it reaches a practically constant value. The course of such a "racemisation" is represented graphically in the accompanying diagram.

The ordinates represent specific rotation values and the abscissae the number of hours the reaction had been allowed to proceed. The solutions were made in semi-normal sodium hydroxide and contained about 7 per cent. of protein (see experimental part).

The points indicated by small circles represent a series of specific rotations, determined on a solution of euglobulin in $N/2$ alkali (7·35 g. protein per 100 cc. solution) at different intervals during the period of racemisation. A second series of readings on a solution of pseudoglobulin in $N/2$ soda (7·04 g. protein per 100 cc. solution) is represented by the crosses. The diagram shows clearly that the optical behaviour of the globulins in $N/2$ alkali is identical. One curve can be drawn to satisfy equally both sets of points. Not only are the initial and final rotations the same in both cases, but the rate of diminution of the optical rotation, which in all probability is conditioned by intramolecular structure, is identical for both globulins.

If $N/4$ instead of $N/2$ alkali be employed as a solvent for the proteins, a rather different optical behaviour becomes apparent. It is found that in each case, using solutions containing about 7 per cent. protein, the specific rotation sinks from an initial rotation of about $-75°$ to a final constant value of about $-66°$, as compared with a final value of about $-37°$ in $N/2$ alkali. In other words, the reaction does not proceed to anything like the same extent in $N/4$ alkali as it does in $N/2$ alkali. The significance of this difference is not quite clear as yet; it is possible, however, that this behaviour may indicate the existence of a definite stage in the racemisation process. Acidification at this stage causes the separation of a flocculent precipitate, whereas in the case where the stronger alkali has been employed, addition of dilute acid only occasions a very slight flocculation. It will be interesting to study in greater detail the process of racemisation under a variety of conditions and to note the influence of differing protein and alkali concentrations on the course of the reaction. For the present, however, it is sufficient to note that the behaviour of the two globulins in respect of their optical rotations is identical in $N/4$ just as it is in $N/2$ alkali.

An explanation of the behaviour of such protein solutions in suffering a gradual diminution of their specific rotations has been put forward by Dakin [1912]. According to this view, the racemisation of proteins by dilute alkali at low temperatures is due to a keto-enol tautomerism of the $=CH-CO-$ groups in the protein complex.

$$>CH-CO-NH- \longrightarrow >C=COH-NH-.$$

Wherever this change occurs, the asymmetry of that particular grouping will be destroyed. Consequently, if the racemised protein be hydrolysed, it will be found that certain of the amino-acids are totally inactive, whilst others are fully or partially active. It is extremely probable that the optical properties of the amino-acids thus isolated depend upon their positions in the peptide chains. Those amino-acid components of the protein molecule, whose carboxyl groups are free and which therefore may occupy terminal positions at the ends of peptide chains, will be incapable of racemisation, whilst those which are attached to the protein molecule by condensation of their carboxyl groups with the amino-groups of other constituents, and are therefore probably situated within the chains, may undergo racemisation, since with such an arrangement the keto-enol tautomeric change is possible, this change being accompanied by loss of asymmetry of the grouping in question. On the grounds of the absolute stability of racemised caseinogen and caseose against the common proteolytic enzymes, Dakin and Dudley [1913] concluded that the racemisation due to this tautomeric change must be complete for every such group affected in the protein complex. It thus becomes possible to obtain some evidence as to the original positions of the amino-acids within the protein molecule by a study of the optical properties of these acids among the hydrolytic products of the racemised protein.

In the accompanying table are given the optical properties of the amino-acids derived from the hydrolysis of racemised euglobulin and pseudoglobulin. The results obtained in a similar manner by the authors [1915] for the caseinogens of the cow and the sheep are also tabulated for purposes of comparison.

	Racemised colostrum euglobulin	Racemised colostrum pseudoglobulin	Racemised cow's caseinogen	Racemised sheep's caseinogen
Glycine... ...	—	—	—	—
Alanine ...	inactive and *d*	inactive and *d*	inactive (and *d*?)	inactive (and *d*?)
Valine	*d* and inactive	*d* and inactive	*d* and inactive	*d* and inactive
Leucine ...	*l* and inactive	*l* and inactive	*l* and inactive	*l* and inactive
Tyrosine ...	inactive	inactive	inactive	*l*
Phenylalanine...	inactive	inactive	inactive	inactive
Proline... ...	*l*	*l*	*l*	*l*
Aspartic acid ...	inactive	inactive	inactive	inactive
Glutaminic acid	*d* and inactive	*d* and inactive	inactive	*d* and inactive
Arginine ...	inactive	inactive	inactive	inactive
Histidine ...	inactive	inactive	inactive	inactive
Lysine	inactive	inactive	inactive	*d*

It will be noted that the amino-acids from racemised euglobulin and pseudoglobulin exhibit no differences whatever. This is regarded as a further confirmation of the structural identity of the two proteins.

The inactivity of the "hexone bases" is noteworthy; it would seem that these diamino-acids in the globulins (as also in cow's caseinogen) are situated entirely within the peptide chains. This fact would appear to lend support to the view sometimes put forward, that they represent the actual "foundation stones" on which the protein molecule is erected. Their basic character would naturally fit them for filling such a function.

It is also curious to notice that, though globulin contains about 3·5 per cent. of glycine, yet in neither case was this amino-acid found among the products of hydrolysis of the racemised globulins. This may possibly be due to the glycine being split off during the racemisation with loss of ammonia, since a certain amount of hydrolysis always occurs simultaneously with racemisation. If this be true, it would seem to indicate that the total glycine in globulin is so situated within the molecule, that it is split off with extreme ease by the action of hydrolysing agents. In this respect, globulin exhibits a markedly different behaviour from gelatin, as Dakin [1912] was able to recover about 17 per cent. of glycine from the products of hydrolysis of racemised gelatin. Eu- and pseudo-globulin behave identically in this respect; so that this fact is also consistent with the idea of their structural similarity.

In comparing the optical properties of the amino-acids derived from the hydrolysis of racemised cow's caseinogen and colostrum globulin, one is struck by the close similarity displayed, suggestive, it may be, of a near structural relationship between racemised caseinogen and racemised globulin. Such a kinship is not altogether impossible, in view of the fact that globulin appears to lose glycine during racemisation, whereas caseinogen, in like circumstances, suffers a loss of phosphate content.

In conclusion, reference may be made to an interesting confirmation of the results arrived at in this investigation, regarding the identity of the globulins in colostrum, which is furnished by work carried out in this Institution by Crowther and Raistrick [1916]. They analysed euglobulin and pseudo-globulin by the method of van Slyke and were unable to detect any differences in the amino-acid content of the two proteins. The results of their analyses demonstrated further that the total globulin of milk was identical in composition with that of colostrum.

Experimental.

Preparation of euglobulin and pseudoglobulin from cow's colostrum.

Colostrum, which is the fluid secreted by the mammary glands in the earliest stages after parturition, is eminently suited to be the starting point for the preparation of globulin. The colostrum secretion is distinguished by its richness in protein material, which may at times amount to as much as 18 to 20 per cent. of the total secretion. Of this, 4–5 per cent. usually consists of caseinogen, 0·7–1·5 per cent. of albumin and 6–12 per cent. of globulin. This is in striking contrast to the ordinary milk secretion which usually contains about 3·0 per cent caseinogen and 0·4 per cent. albumin, whilst globulin is only present in very small amounts. The preponderance of globulin in the initial secretion rapidly disappears with the development of mammary activity and the colostrum assumes the characteristic composition of milk often within about 48 hours of calving [Crowther and Raistrick, 1916].

The first step in the preparation of the globulins was to precipitate the caseinogen by means of dilute acetic acid. The best conditions for precipitation were found to be as follows.

The colostrum was added to dilute acetic acid containing 2 cc. of glacial acetic acid per litre; for every litre of colostrum, it was found advisable to use 2 litres of the dilute acid. The mixing was carried out in a tall glass jar and the liquid was stirred vigorously during the addition. The caseinogen, carrying along with it the bulk of the fatty material, immediately separated out.

After allowing the caseinogen precipitate to settle, the clear liquid, containing the globulins and albumin in solution, was siphoned off. A further quantity of globulin liquor was obtained by centrifuging the residual slime of caseinogen and fat.

The solution was next passed through a folded filter paper and made exactly neutral by the addition of the requisite amount of dilute soda. The large volume of liquid was then reduced somewhat by concentration in a vacuum pan at a temperature of 40–45°, and a sufficient quantity of magnesium sulphate was then stirred into the concentrated solution to ensure complete saturation. This effected the separation of the globulins, which were filtered off on a large Buchner funnel. The filtrate was preserved with a view to isolating the albumin for further work.

The mass of mixed globulins was dissolved in water and resalted out by means of magnesium sulphate, in order to render the separation from albumin

more complete. The globulin so obtained was once more dissolved in the minimum amount of water and the solution was thoroughly dialysed against running tap water, in the initial stages, and finally against distilled water. During this process, the euglobulin separated out in a gelatinous form from the solution. The pseudoglobulin, which is soluble in distilled water, remained in solution.

The dilute solution of pseudoglobulin, after filtration, was concentrated *in vacuo* at a temperature of 40–45°, until a content of about 17 per cent. protein was attained. Emulsions of euglobulin (after thorough washing with distilled water) were prepared of a similar strength by stirring the protein into water containing a little common salt in solution. The advantages of working with such protein preparations, instead of employing the dried materials, were twofold. Firstly, we were enabled to avoid all processes which might have exerted a "denaturating" effect on the protein (*e.g.* excessive heat, treatment with alcohol, etc.). Secondly, it was possible, after carrying out a nitrogen estimation on such preparations, to make up quickly and without difficulty, solutions of any desired strength in alkali. This was a point of some importance, as it was required to determine the optical rotations of such solutions with as little delay as possible.

Rotatory properties of euglobulin and pseudoglobulin in dilute alkali.

If a 10 per cent. solution of euglobulin or pseudoglobulin be made in $N/2$ caustic soda solution, agglutination occurs almost immediately, the solution thickening to the consistency of a jelly. The jelly-like mass softens after 15 to 20 minutes; but this occurs much more readily if the flask containing the solution be placed in an incubator at 37°. In quite a short time, a clear solution of the globulin is obtained, which can be filtered without difficulty.

It will be noted that both globulins display the same physical changes accompanying their solution in dilute alkali.

In the following series of determinations, solutions of globulin of a 7–8 per cent. concentration in $N/2$ alkali were employed. At higher concentrations than this, it was found that the change from the jelly phase to the clear filterable solution was slower than desirable, in view of the importance of determining the rotation as soon as possible after making the solution.

The method of preparing such a solution was as follows. 10 cc. of $5N/2$ caustic soda solution were run into a 50 cc. graduated flask. After diluting this with distilled water, a measured amount of the globulin preparation, containing about 3·5 g. of protein, was added quickly and the bulk made up

to the 50 cc. mark by the addition of distilled water. During the mixing process, the contents of the flask were shaken vigorously. The flask was then placed in the incubator, and after about two hours, the solution was filtered and the optical rotation determined in a decimetre tube. The exact concentration of the filtered solution was determined by a Kjeldahl estimation on 5 cc.

The rotation of the solution was determined at regular intervals, until it had attained a constant value. The two tables which follow give the results of comparative trials with the two globulins in $N/2$ caustic soda solution. It will be noted that the data, which have been plotted in the diagram already referred to, failed to reveal any differences in the structural make-up of the globulins. Such slight discrepancies as may exist fall well within the limits of experimental error. In both cases, the specific rotation fell rather rapidly in the first stages of racemisation; but the rate of diminution became slower later on, until finally the rotatory value became practically constant.

I. Pseudoglobulin (7·04 g. in 100 cc. $N/2$ alkaline solution).

Time (in hours)	Rotation in 1d tube	$[\alpha]_D = \frac{\text{Rotation}}{\text{Concentration}}$
2·25	−5·22	−74·15
3·5	−5·18	−73·57
5	−5·00	−71·02
22·5	−4·15	−58·95
30·5	−3·83	−54·40
47	−3·45	−49·00
94	−3·03	−43·04
120	−2·93	−41·62
142	−2·87	−40·77
170·5	−2·73	−38·78
194·5	−2·68	−38·07
215·5	−2·65	−37·64
262·5	−2·65	−37·64

II. Euglobulin (7·35 g. in 100 cc. $N/2$ alkaline solution).

Time (in hours)	Rotation in 1d tube	$[\alpha]_D = \frac{\text{Rotation}}{\text{Concentration}}$
2·5	−5·46	−74·28
5	−5·18	−70·48
22	−4·23	−57·55
52	−3·45	−46·94
72·5	−3·20	−43·54
95	−3·08	−41·91
120	−3·02	−41·09
167	−2·86	−38·91
190·5	−2·76	−37·55
336	−2·76	−37·55

Before leaving this part of the subject, it may be pointed out that the method outlined above should prove of value in future investigations, which may be designed with a view to establishing the identity or non-identity of related proteins. Its relative simplicity and the small amounts of material required are points in its favour. Its operation does not involve the length of time and the vast amount of labour, which are required by many of the ordinary methods of protein investigation. Moreover, it may be made a very exhaustive test; since if two proteins are to be pronounced structurally identical, then they must not only display the same optical behaviour under one set of conditions, but must continue to do so when the concentration of the protein and the strength of the alkaline solution are varied.

Isolation of Amino-acids.

For the purpose of isolating the amino-acids, 10 per cent. solutions of the globulins in $N/2$ NaOH were digested at 37° for 8–10 days. The rotation in a decimetre tube fell during this period from about $-7{\cdot}5°$ to a constant value of about $-4°$.

Tyrosine. 45 g. euglobulin and an equal weight of pseudoglobulin were racemised separately. The alkaline solutions were hydrolysed with hydrochloric acid, the solutions filtered and evaporated *in vacuo* at 45°. Water was added to the residues, and the solutions were once more evaporated under these conditions. This process was repeated until the bulk of the hydrochloric acid had been removed. The residues were subsequently dissolved in water, boiled with the addition of animal charcoal and filtered. After making the solutions slightly alkaline by means of ammonia, they were concentrated and the tyrosine was allowed to crystallise. The two specimens of crude tyrosine were filtered off, washed with a little hot glacial acetic acid and then dissolved in dilute hydrochloric acid. After treatment with animal charcoal, the solutions were neutralised with ammonia. On cooling, tyrosine separated out in characteristic form. The two samples were further repeatedly crystallised from slightly ammoniacal solutions, in order to remove completely traces of cystine. They were then dissolved in 4 per cent. hydrochloric acid and their optical rotations determined. They were both found to be inactive.

The mother liquor, from which the crude tyrosine had crystallised, was acidified with hydrochloric acid and added to the acid solution of mixed amino-acids for investigation by the esterification method.

Arginine, histidine and lysine were isolated by the method of Kossel and

Kutscher [1900]. For this purpose, 135 g. of euglobulin and 150 g. of pseudoglobulin were racemised separately. Arginine and histidine were in both instances inactive. The lysine fractions displayed definite *laevo* rotations when first isolated, owing to contamination with *l*-proline. It was found possible to remove the latter by fractional precipitation of the phosphotungstates, but a more convenient method was as follows. The copper salts of the mixture were prepared in the dry, powdered form. The latter preparation was then thoroughly extracted with absolute alcohol, in which the proline-copper salt was readily soluble. The residual insoluble lysine-copper salt was dissolved in water, acidified slightly with dilute sulphuric acid and decomposed by means of sulphuretted hydrogen. After such treatment, the lysine preparations from both sources were found to be totally inactive, whereas the fraction obtained from the decomposition of the proline-copper salt possessed a decided *laevo* rotation.

The large volume of acid solution, obtained in the above isolation of the diamino-acids after the filtration of the phosphotungstate precipitates, was made almost neutral by the addition of powdered commercial baryta. After filtering, the solution was evaporated to a small bulk in a vacuum pan at 45°, and the remaining sulphuric acid was removed quantitatively from the concentrated liquid by means of baryta. The solution thus obtained was acidified with hydrochloric acid and utilised to augment the yields in the investigation of the amino-acids by the esterification method.

200 g. of euglobulin, and 240 g. of pseudoglobulin, were racemised separately in the manner already described and then hydrolysed with hydrochloric acid for the amino-acid separation by the esterification method. To the acid solutions were added the solutions of amino-acids recovered from the tyrosine and diamino-acid separations. Levene's modification of Fischer's method was employed [Levene and van Slyke, 1909].

Glycine. As already mentioned, no glycine was found among the amino-acid preparations resulting from the hydrolysis of racemised euglobulin and pseudoglobulin. The fraction of crystals containing alanine and valine, where the glycine, if present, should occur, was fractionally crystallised, and the percentage of nitrogen in the more soluble portion of the crystals was determined. After carrying out this procedure repeatedly, the percentage of nitrogen increased to a constant value of 15·7 per cent., which is the nitrogen content of pure alanine, whereas that for glycine is the much higher value of 18·7 per cent.

The absence of glycine was confirmed by our inability to obtain crystals

of the characteristic picrate from the alanine fractions by the method of Levene [1906].

Alanine. Specimens of pure *i*-alanine were obtained by fractional crystallisation from both globulin sources. In both cases, it was also found possible to recover other fractions containing alanine and valine, in which the rotation was *dextro*. They consisted of mixtures of *d*-alanine with *i*- and *d*-valine.

Valine was isolated in mixtures of *i*- and *d*-valine, containing as much as 70 per cent. of the active form, from both racemised globulins. In addition to this, specimens of *i*-valine were isolated in each case, and the *dextro* variety was found to occur in leucine-valine mixtures and in valine-alanine fractions.

Leucine. Both racemised globulins gave leucine in the pure state almost inactive, whilst in each instance, fractions of *l*-leucine containing only small amounts of *d*-valine were isolated.

Phenylalanine was isolated in the pure state. The specimens from both racemised globulins were inactive.

Proline. In both cases, the proline was strongly *laevo* rotatory, but displayed the partial racemisation which always occurs as a result of the process of separation. It was probable that in neither case had any racemisation taken place previous to hydrolysis.

Aspartic acid was isolated in the inactive form from racemised euglobulin, as well as from racemised pseudoglobulin. In neither case was any of the active variety detected.

Glutaminic acid. A specimen of glutaminic acid containing about 44 per cent. of the *dextro* variety was isolated from the hydrolytic products of racemised euglobulin. Racemised pseudoglobulin yielded an acid, which contained about 37 per cent. of the active form. These results were sufficiently close to be confirmatory of the identity of the two globulins, especially in view of the fact that a partial racemisation of glutaminic acid is brought about as a result of the hydrolysis and the subsequent process of isolation.

Summary.

It has been shown that:

1. Euglobulin and pseudoglobulin of cow's colostrum display the same behaviour in respect of their optical properties, when dissolved in $N/2$ and $N/4$ alkali.

2. The optical properties of the amino-acids derived from the hydrolysis of racemised colostrum euglobulin are identical with those of the corresponding acids from hydrolysed racemised colostrum pseudoglobulin.

These results are in accord with the findings of Hartley [1914] and Chick [1914] on the corresponding globulins of ox-serum, and of Crowther and Raistrick [1916] on the colostrum globulins; they are confirmatory of the hypothesis, that the two globulins are identical, at least as far as the protein part of their molecules is concerned.

This reservation is necessary in view of the observations of Hardy [1905] and Haslam [1913] on the phosphorus content of globulin prepared from ox-serum. Haslam prepared pseudoglobulin free from phosphorus and showed that euglobulin contained about 0·1 per cent. of phosphorus, about half of which was extractable with alcohol and ether.

The results given by Hartley on the one hand, and Crowther and Raistrick on the other, amply demonstrate that such slight differences are limited to the non-protein ingredients of the globulin preparations. Chick [1914], as a result of her investigations on the serum globulins, expressed the opinion that the phosphorus content of euglobulin was due to the association of the protein with a serum-lipoid. The incomplete extraction of the fatty substance containing the phosphorus in the euglobulin preparation was probably due to the state of aggregation of the protein and not to the phosphorus constituting an integral part of the euglobulin protein molecule.

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XXIX. ON THE THEORY OF GELS.

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In the present paper it is proposed to develop the theory, previously outlined [1917, 1, 2], that the gelation of the natural emulsoids is a crystallisation process, which follows from von Weimarn's theory in consequence of their small coefficient of diffusion and the considerable viscosity and molecular aggregation of their sols. The properties of gels result simply from the structure thus involved. In certain cases, such as the so-called thermal anomaly of gels, and the heat of imbibition of gelatin, where the recorded facts are at variance with the theory, previous observations have been shown to be in error and results obtained in agreement with the values required.

1. On the reversible sol-gel transformation.

Perhaps the theory of gel structure which has found most favour of late is that adopted by W. Ostwald which considers gels to be composed of two liquid phases persisting from the sol stage. Hatschek's mathematical investigation [1917] showed, from the stress-elongation curve, that this hypothesis is untenable. The assumption is implied that gels have a solid disperse phase, consisting of discrete particles, or of aggregations of such particles. We thus return to the ideas comprehended in the earliest theories of gel structure. M. L. Frankenheim [1835] considered that gels were composed of aggregates of small crystals with pores between, which might account for the low density, easy solubility and elasticity of such bodies. K. von Nägeli [1858] thought that a distinction should be made between distensible substances like gels and merely porous bodies. He supposed that gels consisted of molecular complexes or micellae with crystalline properties, separated by skins of water and forming meshes (or interstices) in which the water was contained by molecular attraction. Later [1862, 1] he wrote "Organised substances are composed of crystalline doubly refractive molecules aggregated from many atoms but which lie closely in definite arrangement with one another. In the moist condition, in consequence of the unbalanced attraction, each is

surrounded with a skin of water." It has recently been suggested by more than one writer that this theory agrees most nearly with the properties of gels, and further study fully confirms this view. Although von Nägeli used the term "meshes (Maschen)" he may have referred merely to the interstices in the micellae. This appears probable in the light of a quotation given below. However, from this time, a number of unsuccessful attempts have been made to devise a geometrical framework which would account for the properties of gels. O. Bütschli's extensive researches [1892, 1896, 1898, 1900] on foams and gel structure are well known. As a result of microscopic examination he came to the opinion that the properties of gels might be explained upon the basis of a honeycomb structure in which the walls were permeable to liquids because of their extreme thinness, although he admitted that the walls might be porous.

W. Pauli [1902] objected to Bütschli's assumption that the use of alcohol and chromic acid in his experiments to make the microstructure visible did not alter the structure of the gels. Von Weimarn [1914] also pointed out that the honeycomb appearance is probably an optical effect due to the overlapping of grains. From a study of imbibition Arisz [1915] came to a decision adverse to a honeycomb structure.

Moreover, if such a structure is dependent on the directive forces of crystallisation, a different arrangement would seem to be implied for each gel. Such differences in the structure of microscopically visible crystal aggregates were indeed found by Zsigmondy and Bachmann [1912] in their work on the gelation of sodium and potassium oleate, palmitate and stearate sols. It seems probable therefore that a single network will not account for the elastic and thermal properties of different gels.

The hypothesis of a network is not supported by the viscous behaviour of gels which gradually mould themselves to the shape of the containing vessel. The gradual motion of small solid masses through gels, under the influence of gravity or of diffusing streams of particles, leaving the gel apparently unchanged, has frequently been noticed during recent work. Moreover suspended cylinders tended to become ellipsoidal.

Further evidence against a net structure is afforded by the lens-shaped form of gas bubbles generated in gels. This phenomenon was investigated by Hatschek [1914] who found that the bubbles place themselves at right angles to pressure and parallel to tension applied to the gel. By measuring the angles between pairs of bubbles in 10 per cent. gelatin, Hatschek found certain values approximately repeated a number of times, although other

measurements varied enormously. A similar appearance may be obtained in 1 per cent. agar by immersing the free gel containing $N/5$ Na_2CO_3 in $N/4$ CH_3CO_2H. Planes of cleavage are clearly suggested. The simplest structure that would conform approximately to the conditions is that of piled shot. This agrees with the globulitic structure observed ultramicroscopically by Bachmann [1911]. Examination of gelatin gels of from 1–6 per cent., from which the liquid had been expressed, showed irregular flocks of separate granules with clear spaces intervening. Increasing concentration reduced the size of the grains (as would be required by von Weimarn's theory) and, at the same time, the empty spaces gradually disappeared, until, at about 6 per cent., the solid phase filled the whole field and the single granules could be no longer differentiated. Agar and silicic acid gels showed a similar globulitic structure.

From these considerations it seems probable that the solid phase of gels consists of discrete granular particles. Since it is now recognised that the distinction between the suspensoids and emulsoids is rather a question of the affinity between the two phases than of the solid or liquid state of the disperse phase, there seems to be the less reason for refusing to extend von Weimarn's theory to the natural emulsoids and regard their gelation as a crystallisation process.

A connection between the precipitation of colloids and the process of crystallisation is certainly indicated by the fact that both suspensoids and emulsoids exhibit the phenomenon of supersaturation. W. Ostwald [1897] has shown that there is a minimum quantity of a substance which is capable of causing the crystallisation of a metastable solution. In the case of sodium chlorate this is equal to about 10^{-10} g., corresponding to a cube with a side of several microns. This is well above the size of the disperse phase in sols. The existence of sols of greater concentration than the ordinary solubility of the substance concerned is therefore in harmony with the theory of supersaturation. The phenomena of supersaturation have frequently been observed in the case of sols. Thus gold separates spontaneously from gold sols of more than a certain concentration and the precipitation may be accelerated by adding a few drops of a second solution of colloid gold. Garrett [1903] found that gelatin sols gelate more rapidly if some solid gelatin, prepared by rapidly cooling the same sol, be added. The spontaneous coagulation of rubber emulsions is hastened by the addition of solid rubber. Additional evidence is furnished by the temperature variation of the concentration of the liquid expressed from gels. In the liquid from a 2·3 per cent. agar gel

Hardy [1900] found from 0·09 per cent. of agar at 5° to 0·47 per cent. at 36°. This is corroborated by the influence of concentration on the temperature of gelation [Pauli and Rona, 1902; Rohloff, 1907; Levites, 1908].

The literature of organic chemistry abounds in descriptions of definite substances which occur in the gel form. Frequently, by suitable choice of solvent, these have also been obtained as well defined crystals. The following examples are taken at random. The sodium salt of acrylic acid occurs as microscopic lenticular needles. The boiling saturated aqueous solution sets on cooling to a jelly which shows no structure under the microscope [Linnemann, 1874]. Glycyrrhizic acid, $C_{44}H_{63}O_{18}N$, which occurs in licorice root, crystallises from alcohol as light yellow glistening leaflets, which are the better formed the slower the separation from alcohol. It is characteristically little soluble in water at ordinary temperatures. In boiling water the substance is easily soluble and the solution forms on cooling a light wine coloured jelly. By mixing 1 g. of glycyrrhizic acid with 100 cc. of water a jelly is formed, after some hours standing, of such a consistency that the vessel may be inverted without loss [Habermann, 1879].

The gels of camphorylphenylthiosemicarbazide, investigated by Hatschek [1912], led him to suspect a crystalline structure formed by growth of amicrons into a network: a conclusion which he hesitated to accept on account of the mathematical difficulties. These gels appear to be typically elastic, and are produced (1) when a hot alcoholic solution of the substance up to about 5 per cent. is suddenly cooled, (2) from the gradual or rapid cooling of toluene solutions of similar concentration, or (3) by pouring an alcoholic solution into petroleum. The latter method gives gels with a concentration as small as 1 : 400. The solubilities in alcohol, toluene and petroleum are respectively 0·5 per cent., 1 per cent. and very small. Von Weimarn's theory affords a ready explanation of such cases. From the examination of a very large number of precipitates von Weimarn showed that the degree of dispersity in which a substance occurs is regulated by the number of crystallisation centres, N, produced according to the formula $N = KP/L$, where L is the solubility of the substance, P the excess concentration $(C - L)$, and K is a factor which depends on the degree of aggregation of the reaction components in solution and the viscosity of the reaction medium. A sol or gel is formed according as a large value of N is produced from a small value of L or a large value of P. Intermediate values of L and P give rise to micro or macro crystals. By actual inoculation of supersaturated solutions, von Weimarn has shown that in every stage, except the two extremes, the solid phase is crystalline.

He infers that the disperse phase of such sols and gels is likewise crystalline though the particles are too small to act as nuclei.

Considering the general case of a substance of high molecular weight and very low diffusivity, let this be treated with a liquid in which its solubility, L, shall be small in the cold and increase considerably, but not too rapidly on heating. Then, on applying heat, the process will be reversible and the Noyes-Nernst formula for the velocity of solution, $V = \frac{D}{\delta} S (L - C)$, will hold. Since L is very small, V will be very small indeed, and the sol stage will persist. Further heating will lead to the gradual breaking up of the aggregated particles of the sol and an approach to true solution. If the stage of single molecules is reached, this may be regarded as a true solution, although the diffusion constant will be that of a sol. Notwithstanding the size of the molecules, the system may be looked upon as homogeneous and von Weimarn's formula applied. On cooling, with reduction of L, aggregation will commence, but the velocity of crystallisation, $V = \frac{D}{\delta} S (C - L)$, will be a minimum, so that diminution of L will give a larger and larger P and a still greater value of N, since $\frac{dN}{dL} = - K \frac{C}{L^2}$. The velocity of crystallisation will also increase, and, if the laws of supersaturation apply, spontaneous crystallisation will eventually take place. Since N must necessarily be very large, from the large values of P and K, as well as the small value of L, a gel will be formed. Owing to the small velocity of crystallisation, gelation will however take place slowly. The formation of a gel will therefore be dependent on the concentration, the thermal variation of solubility and the factor K, which represents the degree of association and viscosity of the sol.

The natural emulsoids such as gelatin and agar appear to differ from the organic substances considered above, merely in the fact that they do not ordinarily occur in the crystalline form, though von Weimarn [1910] indeed claims to have obtained crystals of gelatin. Although little is known as to the true solubilities of these substances, there seems to be no reason for regarding them as exceptions to the general case. Actually their known properties are exactly such as to bring them within its scope. They form sols of considerable concentration, aggregation and viscosity. Heating at constant temperature decreases their viscosity, while cooling increases it [Levites, 1908], changes which are due to variation in the number as well as the size of the disperse particles and are thus seen to be in accordance with Einstein's formula. Osmotic pressure varies in the opposite sense.

The heat coagulation of albumin may be explained on the supposition of

the chemical production of an insoluble compound. In this case the gelation should be, and actually is, irreversible. Silicic acid behaves very much in the same way as the typical case of aluminium hydroxide investigated by von Weimarn. The gel is formed immediately when the equivalent amount of hydrochloric acid is mixed with a moderately concentrated solution of potassium silicate. This is due to the sudden production of a highly aggregated insoluble form of silicic acid, owing to the concentration, aggregation and viscosity of the reaction medium. When, however, the silicate solution is slowly stirred into the mineral acid, thus keeping down the value of K, probably a true solution of silicic acid is formed at first, as is indicated by its high diffusivity.

While the foregoing cannot be considered as a rigid proof of the hypothesis, it seems permissible, in the absence of evidence to the contrary, to regard the reversible sol-gel transformation of the natural emulsoids as due to their large value of K, together with their low diffusion constant, which, by retarding crystallisation, allows the accumulation of a sufficiently large excess concentration to cause gelation on cooling. The theory receives confirmation from the fact that all the properties of gels are found to be consistent with the structure thus conditioned, and from the verification of certain new facts indicated thereby.

Similar considerations would appear to account for the occurrence also of resins and of glass. Indeed, from experiments on the introduction of metals into the surface layer of glass, Rhemberg is convinced that we should regard this as a substance full of ultramicroscopic pores. Various definite organic substances are easily obtained in either the crystalline or resinous form at will. Piperine crystallises from alcohol in monoclinic prisms which melt at 132° and set on cooling to a light yellow transparent resin. On heating to 100° the resin loses its transparency, becoming nearly opaque, like sulphur, owing to its transformation into a mass of microscopic crystals which undergo no further change until they melt at 132°. On cooling the resin is formed as before. If this is kept for about ten days, yellow sulphur-like spots of microscopic crystals begin to appear and very gradually extend throughout the mass. But after heating to 180° for an hour, this change does not begin in $2\frac{1}{2}$ years [Madan, 1901].

2. On spherites in relation to gel structure.

Before proceeding to the examination of the properties of gels, it seems worth while to inquire into the actual form of the disperse phase. As mentioned above, ultramicroscopic evidence shows a globulitic structure, but since the specific surface of gels must be extremely large, it seems probable that the individual grains are themselves aggregated. Von Nägeli was unable to say whether his micellae were identical with spherites[1], but the evidence now available points to this conclusion. W. Ostwald [1891] states that microscopic observation of the process of crystallisation shows that (at least in the case of substances of low melting point) small spherical bodies first appear. Only after they have attained a certain size do they assume crystalline shape. Linck [1839] first observed that substances deposited from solution appear originally as small droplets. Frankenheim [1860] describes the appearance of globules in a drop of saturated aluminium sulphate in water made turbid by addition of alcohol. However, Richards and Archibald's cinematographic study [1901] of the first appearance of growing crystals failed to show the occurrence of globules in any of the cases investigated, and it is doubtful whether a transitory spherical phase appears, except in the case of substances of low melting point.

Spherites were first studied by Brewster [1853] who observed their production when solutions were allowed to crystallise under pressure between glass plates. He believed the pressure to be an essential cause of the phenomenon, but noticed the contributing effects of quick cooling and increase of viscosity. Rainey [1858] obtained microscopic spherites of calcite by allowing the slow mixing by diffusion of solutions of $CaCl_2$ and Na_2CO_3 in gum water. Kuhlmann, in 1863, pointed out the important influence of increasing the viscosity of the solution. With a view to slowing the crystallisation, he added such substances as gum and gelatin. When crystallisation took place sufficiently rapidly, the crystal particles which first appeared quickly grew into spherites. The structure of such bodies, occurring naturally in Acetabularia, was described by von Nägeli [1862, 2]. "The smallest spherites (up to about 40 μ) were perfectly spherical.... In the unchanged state... they appear almost like oil drops or starch grains, though more glass like.... Often they show clear banding. The bands have a very regular and quite concentric relation to the surface.... This stratified arrangement proves that the spherites

[1] In this paper the term "spherite" is used to include spherical aggregates, spherical crystals, spherocrystals, spherulites, globulites and all such approximately spherical crystalline aggregations, the origin and structure of which appears to be similar.

grow by additions to their surface.... They appear as clear stripes which are mostly at exactly equal distances apart. Besides the concentric bands, frequently there appears a radial striation.... The substance is divided thereby into meshes of more or less quadratic form.... The ringed structure of the spherites is very like that of starch grains and cell membranes.... By drying they become dark, since all their tiny meshes become filled with air." Von Nägeli also observed the beautiful brushes and rings which these crystal aggregates show in polarised light. Frankenheim remarks on the periodic growth of the spherites, which he considers the cause of the concentric rings like the yearly rings observed in a sawn tree stem. "After a time the process becomes slower, sometimes part will redissolve, but soon the exhausted liquid recovers itself, and growth begins anew, soon again to be interrupted." Frankenheim also recognised the relation between the concentric zonal structure frequently observed in minerals and the periodic growth of spherites. Dallas [1875] obtained beautiful spherites, especially of copper sulphate, lead acetate and morphine hydrochloride, by spreading the solution on glass plates and allowing rapid evaporation. Vogelsang [1875] studied the crystallisation of sulphur in carbon disulphide containing thickening material. The substance first appeared in the form of supersaturated droplets as Frankenheim had observed. These gave up more and more of the contained liquid and gradually solidified to isotropic spheres, or spherites. Reinisch, in 1884, obtained magnificent results with double salts of ammonium and sodium, prepared by dissolving 1 g. of ammonium sulphate and 2 g. of crystallised sodium sulphate in 80 parts of water and adding 10 per cent. of nitric acid. With a magnification of 20 the spheres were 2 cm. in diameter. They consisted of needles arranged radially with a notched edge, showed a grey shadow cross and three wide concentric colour rings, but only as long as the crystals were still moist. After drying the crystals appeared silvery white. Very beautiful results were obtained with erbium yttrium sulphate without the addition of nitric acid.

Numberless examples are known of organic compounds which crystallise as spherites. These are usually substances of high molecular weight, or such as are probably much aggregated in solution. Inulin is deposited from water as spherites only when a saturated solution is slowly crystallised. Quick evaporation forms amorphous crusts. Evaporation of a drop of an aqueous solution on glass leaves a transparent horny fibrous mass. If the evaporation is slowed, as by placing a coverglass with a hanging drop of the inulin solution upon a moist cardboard frame, good doubly refracting spherites are obtained

[Leitgeb, 1888; Fischer, 1902]. The growth of spherites of amylodextrin was observed by Syniewski [1902]. "If a drop of the [formaldehyde] solution is placed on a microscope slide, covered with a cover glass, and brought under the microscope, after an hour the commencement of the crystallisation is seen. Needle-like crystals shoot at first to grouped star-like bundles. After a time these bundles become composed of regular concentrically arranged layers of extraordinarily fine needles directed towards the centre, which then show a thousand structures like starch grains. This imitation of starch grains goes so far that aggregated grains are also seen to originate. All these grains are composed of doubly refracting needles and show beautifully, in polarised light, the well known dark cross of the starch grains." The same author has shown that starch grains consist wholly of a single substance and that the amylocellulose, of which the outer skins of the grains were supposed to be formed, is a decomposition product. Having regard to Bütschli's [1898] preparation of artificial starch grains by crystallisation of starch in presence of gelatin, little doubt remains that the starch grains are themselves identical in origin and structure with spherites.

The production of large crystals by slow diffusion, as through strips of filter paper [Monnier, 1874], or gels [Vesque, 1874], is well known. In 1879 Ord observed the precipitation of calcium oxalate by diffusion of ammonium oxalate through an isinglass gel in an open tube immersed in calcium chloride solution. The precipitate consisted first of growth forms changing into spherites towards the calcium chloride, which must have been hypotonic. The very frequent occurrence of spherites in gels was pointed out by Hatschek and is being studied by the writer. Spherites of various difficultly soluble inorganic salts have been prepared by the diffusion of reagents into gels containing solutes with which they react. Frequently the precipitate formed in the upper regions of the gel, where the reagents are more concentrated, is composed of small needles or tiny regular crystals. As the dilution of the reagents increases and the velocity of crystallisation diminishes, the particles become much aggregated and approach spherical shape, often becoming perfect spheres in the lower nearly exhausted regions of the gel. Among others, good examples of spherites of silver bichromate, copper and calcium carbonates have been obtained. The former are opaque, the two latter substances show black crosses in polarised light, and the larger spheres colour rings. Spherites of manganese polysulphide have been produced over 1 cm. in diameter. These are opaque, but have the same banded structure. They resemble closely the large spheres, several inches in diameter, of magnesian

limestone occurring in the Permian beds at Fulwell Hill, Sunderland, described by Abbott. These results show that the formation of spherites is not induced by quick crystallisation. In all the cases quoted it is, however, reasonable to assume a state of molecular aggregation in solution. The slow diffusion in the presence of protective colloid in gels favours the formation of such molecular aggregates in solution. Supersaturation, especially in viscous media and with bodies of high molecular weight, has the same effect, and since the crystallisation of a substance usually begins from a supersaturated solution, the first appearance of spherical masses would be in accordance with an existing state of aggregation. Consequently it may be doubted whether the "droplets" frequently recorded are invariably liquid. If they were so, the cause of their further development into spherites, instead of crystals, in some cases, would still require explanation. There are, however, many bodies of low melting point which undoubtedly appear at first in the form of liquid drops. From Hardy's [1900] experiments with gelatin sols treated with alcohol, this may possibly be the case in the gelation of gelatin. In accordance with Masson's [1891] theory of solution, gelatin may melt in water at a lower temperature than that of complete miscibility, below which there would exist two phases, one of melted solid containing a little water, and the other of water saturated with gelatin. The velocity of crystallisation of gelatin is so slow that even the melted solid phase may form spherites. In any case aggregation in solution appears to be a principal cause of the formation of spherites. Another important factor must be the viscosity of the liquid medium, which restricts the movement of the unwieldy molecules or molecular aggregates. Under the influence of the feebler forces of crystallisation, consequent upon the size of the particles, these have the less opportunity to pack themselves into regular crystals.

From the rapidity of the formation of the layers, Leitgeb [1888] has shown that they cannot be influenced by outside causes, but must be due to changes in the concentration of the solution in the immediate neighbourhood of the crystals. Observation of banded precipitates formed by diffusion of reagents into gels, indicates that the zonal structure is due to adsorption, by the precipitate, of solute from the gel, until the region next the precipitate is exhausted of solute, and the reagent is able to diffuse further into the gel before forming a fresh band [Bradford, 1916, 1, 2]. A similar explanation may be given of the zonal structure of spherites. Since these consist of numerous ultramicroscopic individual particles, their specific surface will be very large. Consequently the solute will be rapidly adsorbed and the

concentration in the region next the spherite reduced. If the concentration fall sufficiently, crystallisation will be momentarily suspended or its velocity reduced until fresh supplies of nutrient material arrive.

These considerations lead to the conclusion that substances of high molecular weight and such as exist in solution in the aggregated form, tend to crystallise in the form of spherites. It is reasonable to suppose, therefore, that the granules seen ultramicroscopically in gels, which must have great specific surface, actually consist of these spherical aggregates.

Spherites of gelatin can easily be produced of microscopic size. A sample of dialysed gelatin precipitated by alcohol, was dissolved in about 100 parts of water and poured slowly into alcohol. The smallest trace of the precipitate brought on to a microscopic slide, showed a mass of innumerable transparent granules, round the edge of which individual grains could be seen. Under a magnification of about 800 the granules appeared perfectly spherical and translucent of about 0·5 to 2 mm. diameter (Fig. 1). No connection between the grains could be seen.

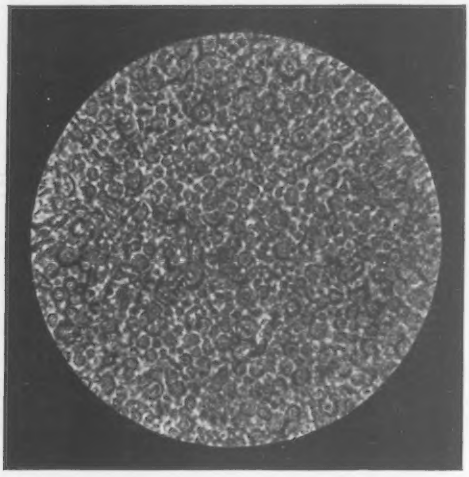

Fig. 1. Gelatin precipitated by alcohol ×800.

The question next arises as to whether the micellae are themselves aggregated to form structures within the gel. On account of the small size of the micellae, the problem is the more difficult to solve. Ultramicroscopic examination of untreated gels gives no indication of such aggregation.

Moore [1915] observed chains of globules in silicic acid sols. The possibility of the existence of microscopic filaments has been suggested [Bradford, 1917, 2]. This is not supported by the viscous properties of gels which permit the movement of solid particles and gas bubbles within them. Aggregation of the micellae is possibly more likely to occur in the surface skins of gels owing to the influence of adsorption and evaporation in increasing the concentration. Hitherto the influence of these skins on the properties of gels does not seem to have received sufficient attention. Moreover, if one region of a sol should gelate before another, adsorption might cause a rhythmic variation in the density of distribution of the gel particles similarly to the bands in spherites. The possibility of aggregation of the micellae within the gel has been investigated ultramicroscopically by Moeller [1916, 1917], who considers that the micellae are united into fibrils, like those in organic membranes, but which are obscured by the thickness of the layers viewed ultramicroscopically. To overcome this difficulty he observes very thin layers of gelating sol upon a microscope slide with a cardioid ultracondenser, and, before gelation, he adds alcohol or some tanning material. In other cases the surface of the gel is treated with the tanning solution, and the appearance observed before diffusion has reached the interior of the gel. These methods are not very different from those employed by Bütschli and others for microscopic examination. Unfortunately both methods are open to great objection. In the first case the addition of precipitating reagents to the sol fundamentally transforms the character of the crystallisation process, and it may be observed that the structures seen were immediately broken up by slightly disturbing the slide during gelation. In the second case, as has been shown, the properties of the surfaces of gels are likely to differ from those of the interior. In both cases the formation of precipitates by the reaction of the tanning reagents, such as chrome alum, with the impurities of the nature of calcium salts in the gel does not appear to be excluded. The presence of such impurities in the gelatin used is confirmed by a photograph showing spherites, produced in the drying gel, exactly resembling the form in which calcium sulphate occurs in gelatin. Such precipitates would undoubtedly be produced in any but the most carefully purified gelatin and might show structures similar to those observed. Moreover, it seems probable, as Wilson has pointed out, that gelatin forms insoluble compounds with alum and chrome tanning material. These would tend to be precipitated in the banded form. The production of similar laminated or striated forms by the crystallisation of salts in gelatin films, is well known, and originates from the action of adsorption and

diffusion as in the Liesegang phenomenon [Bradford, 1916, 1, 2]. From the similarity of the structures seen with Liesegang's rings, Moeller argues, in the second paper, that the latter are due to the pre-existence of laminated structures in the gel. This is certainly controverted by many facts: such as that the distance apart and the width of the precipitated bands can be determined, in a given gel, by a suitable choice of the concentrations of the reacting solutions; that the orientation of the bands is always at right angles to the diffusion path; and that similar banding can be produced in sand columns and filter paper. Moeller also obtains various shrinkage figures, like those previously studied by Liesegang and others, in the surface of gelatin gels by the action of water vapour, hot water, or drying. From these experiments Moeller concludes that, of the α- and β-gelatin of which the gels are composed, the α-gelatin forms fibrils which intertwine to form a network, in the meshes of which is enclosed the β-gelatin, or gelatose, as a structureless mass in solution. The theory is then extended to include all gels. While however the possible existence of fibrils in the interior of gels cannot be definitely excluded at present, it may be considered that the available evidence is insufficient to establish their presence.

3. The structure and properties of gels.

From the point of view of the foregoing, the natural emulsoid gels may be regarded as two phase systems comprising innumerable ultramicroscopic globular crystalline molecular aggregates, or probably spherites, in, and around, which the mother liquor, from which the disperse phase has crystallised, is held by molecular and capillary forces.

Owing to the enormous specific surface, the state of a portion of the liquid phase will differ from that of a free liquid. Assuming with Laplace that the surface tension and intrinsic pressure of liquids may be attributed to the molecular attractive forces, the molecules in the outer skin of liquid, at a liquid-air surface, experience a force directed normally towards the surface which is due to the unbalanced molecular attractions, and, in this surface layer, the intrinsic pressure of the liquid falls rapidly towards that of the atmosphere. Conversely, if it be allowed that solid molecules have usually a greater attraction for liquid molecules than these for themselves, of which there are many indications, it may be supposed that, in a liquid-solid surface skin, the internal pressure tends rapidly to approach the intrinsic pressure of the solid, and may be many times that of the free liquid. The dispersion medium in

gels will exist therefore in at least two states, (1) the adsorbed liquid, *i.e.* that in the skins surrounding the solid phase which will be under an enormously increased pressure and (2) the liquid in the larger interstices of the gel held by capillary forces. Moreover, owing to the action of adsorption and, especially, of evaporation, the boundary surface of gels will be composed of a more or less tough skin which may materially affect the apparent properties of the gel. This skin is much more noticeable in some cases, and varies with the age and previous environment of the gel. Thus gelatin gels have very tough skins, agar gels, much less so, and silicic acid gels hardly noticeable skins. The cohesion of gelatin, agar and silicic acid gels varies in the same way, and since, on cutting the surface of a gelatin gel, the cohesion of the gel is enormously reduced, it seems probable that the cohesion resides largely in the surface skin. Determination of the breaking stress of 15 per cent. gelatin and 2·5 per cent. agar gels gave the small value of about 105 g. per sq. cm. for the latter, as the mean of three experiments, while the gelatin gel did not break with a load of 1282 g. per sq. cm. All these gels were about 30 hours old. It is evident therefore, that, in considering the stress-elongation curve of gels, the elasticity of the surface skin must be taken into account, and the elastic properties of this skin deserve further study.

The vapour pressures of gelatin-water mixtures have recently been studied by Gerike [1915]. Gelatin gels show a slight lowering of the vapour pressure as compared with pure water, and the concentration of the gel is of little importance below 70 per cent. gelatin. From about this composition the lowering of vapour pressure is proportional to the increase in percentage composition of gelatin; at about 94 per cent. there is a second inflexion in the vapour pressure curve. Gerike also found that the nature of the surface has a considerable influence on the behaviour of the gel in an atmosphere of water vapour. These results appear to indicate the existence of the gel water in three states, (1) free water containing solated gelatin, (2) water contained in fine capillaries, and (3) a small mass of more firmly bound water, which may correspond to that in the surface skins around the solid phase and may be called the adsorbed water.

Zsigmondy [1911] has shown that the von Nägeli hypothesis supplies the explanation of the curious hysteresis cycle in van Bemmelen's [1878, 1880] well known vapour pressure isotherms for silicic acid gels. Silicic acid gels dry to a clear transparent finely porous gel, which is practically constant in volume and only permits of volume changes within its elastic limits. The hysteresis cycle corresponds to this constant volume gel.

During the progressive dehydration along the first descending branch of the curve, the gel is decreasing in volume, and the gel capillaries develop menisci at the gel surface, concave to the vapour phase, with increasing curvature corresponding to the fall in the vapour pressure. At the commencement of the hysteresis cycle the radius of curvature of the liquid menisci has reached its lowest value. The radius of curvature has now become approximately equal to the radius of the capillary. Such a meniscus has a maximum pull on the layer of liquid below, in consequence of which the gel walls are drawn together and the gel skeleton as a whole undergoes compression. The tension causes rupture of the liquid in the interior of the gel, and the appearance of the gas phase. The occurrence of innumerable gas bubbles causes the opaqueness which begins at this point. The bubbles are larger than the size of the interstices between the micellae, and envelop a number of particles of the solid phase. Finally, when the emptying of the capillaries is complete, at the lower point of the cycle, the bubbles have all coalesced and the opaqueness has disappeared. These facts indicate an extraordinarily fine structure. Larger discontinuities, of the order of Bütschli's honeycombs, must cause permanent opaqueness.

The ascent of the steeper branch during reimbibition is due to the fact that the unwetted walls develop liquid menisci with greater radii, and consequently higher vapour pressure, than before. The greater water uptake during the last part of this branch corresponds to the enlargement of the capillary volume in consequence of the diminished pressure in the gel occasioned by the gradual flattening of the menisci.

For such a gel the size of the capillaries can be calculated, and, for a reduction of vapour pressure of 6 mm., Zsigmondy finds a capillary radius of about $2{\cdot}5\,\mu\mu$.

This was completely confirmed by Anderson [1914], who replaced the imbibition water by alcohol and benzene, and determined the vapour pressure isotherms in a vacuum apparatus. The curves were of exactly the same form as for water. From the formula $r = \dfrac{2Ts_0}{\sigma p_0 \log p_0/p_1}$ (where r = radius of capillary, T = surface tension of imbibed liquid, s_0 = density of saturated vapour, σ = density of imbibed liquid, p_0 = ordinary vapour pressure of imbibed liquid, and p_1 = vapour pressure of liquid in capillaries) Anderson found the values:

Imbibed liquid	p_1 in cm. Hg.	r in $\mu\mu$
Water	0·84	2·74
Alcohol	2·12	5·58
Benzene	2·9	2·99

These figures indicate that the diameter of the ultimate particles composing the gel micellae is about $5\mu\mu$. Taking the thickness of the surface skin of adsorbed liquid, as calculated from van der Waal's equation, at about $0{\cdot}15\mu\mu$, and assuming a specific surface corresponding to spherical particles $5\mu\mu$ in diameter, the total volume of adsorbed liquid in a so-called 10 per cent. gelatin gel, containing 8·5 per cent. of dry gelatin, would be about 0·8 per cent. This figure agrees fairly with the value calculated from Rodewald's data below.

Organic hydrogels such as gelatin differ from silicic acid gels in the method of their formation. Whereas the gelation of the latter is irreversible, the gelation of the organic gels is reversible. The cause of this difference lies in the solubility of the organic gel substances in the dispersion medium, as has already been shown. This solubility affects the behaviour of the gel on drying. Evaporation leads to gradual deposition of solid in the pores of the micellae as these are pulled together by the increasing curvature of the liquid menisci, with the result that the gel dries to a transparent hard mass. The rehydration process must include the resolation of the deposited excess of gel substance before the full amount of water can be taken up. This is the reason why the organic gel substances will not absorb liquids in which they are insoluble. Bütschli showed, however, how to substitute such liquids for the water in a swollen gel, and Bachmann [1917] utilised his method to obtain constant volume gels of which the vapour pressure isotherms might be studied. The procedure consists in gradually hardening the gelatin, and, at the same time substituting alcohol for the water, by immersing the hydrogel in aqueous alcohol of gradually increasing concentration. In this way a hardened gelatin alcohol-gel is obtained in which the alcohol occupies the same volume as did the water; and the alcohol can be changed for benzene, xylene and other liquids. The vapour pressure isotherms of these hardened gels were of the same type as the van Bemmelen curves for silicic acid, showing a similar hysteresis cycle corresponding to an opaque appearance in the gel. Bachmann also studied a number of other porous substances, such as hydrophane, permutite and wood charcoal, and concluded that there is a series of chemically very different substances with finely porous gel structure, showing similar vapour pressure isotherms, which correspond to the reduced vapour pressure of a liquid inclosed in amicroscopic capillaries. All the curves include a hysteresis cycle analogous to those discovered by van Bemmelen for the gels of copper oxide, iron oxide and silicic acid. The type of the hysteresis cycle is dependent neither on the nature of the imbibed liquid nor

on that of the gel skeleton. Bachmann calculated that the capillaries are 30–300 times smaller than those required by Bütschli's honeycomb structure, which would have the enormous size of 700–800 $\mu\mu$, and could play no part in the reduction of the vapour pressure.

From experiments on the imbibition of pure chemical compounds, Katz [1917] found that the degree of swelling is independent of the chemical nature of the swelling substance. The curves showing the relation between the degree of swelling and the vapour pressure, volume change, and heat of swelling, are similar to the corresponding curves for mixtures of water with non-volatile liquids such as sulphuric acid and glycerol. From this Katz concluded that the swelling process is to be regarded as the formation of a solid solution, and is incompatible with von Nägeli's theory. On the contrary, the question may be asked, whether the causes underlying the phenomena in both cases are not of the same nature, and whether it may not be hoped that the explanation of the similarity will be found in the sphere of action of the intra-molecular forces. This view of the imbibition process is in conformity with Proctor's experiments [1914] which show that the phenomena are dependent on the relation between the osmotic pressures and equality of products of the hydrolysable gelatin salt which is formed and of the external acid solution.

Some indication of the enormous pressures experienced by the adsorbed liquid in gels is afforded by Schroeder's experiments with gelatin [1903] and by Reinke's work with the oedometer [1879]. Working with discs of Laminaria, the latter showed that gels, separated from water by an osmotic membrane, develop pressures, in equilibrium with the water content of the gel, increasing to 41 atmospheres as the water content falls to 16 per cent. of the air-dry gel. Since air-dry Laminaria contains about 20 per cent. of water, the pressures under which the last traces of water are adsorbed must be enormous. It is impossible to ignore the apparent analogy between these pressures and the osmotic pressures of solutions, with the suggestion that both originate in the molecular attractions of the liquid and solid phases or of solvent and solute. Indeed the study of colloids abounds with indications that the difference between true and colloid solutions, and again between these and gels, is merely one of degree, and that the same general laws are applicable to all. In this connection it will be remembered that the close relation between the properties of sols and gels was pointed out by Arisz [1915] who concluded that gels should be regarded as liquids of great viscosity.

Reinke's experiment does not lead to a direct estimation of the pressure

of the adsorbed liquid in gels. More insight is obtained by examination of the phenomena of swelling.

The total contraction of gel and water on imbibition by gelatin has been studied by Lüdeking [1888]. Unfortunately he used air-dry gelatin, which still contains about 15 per cent. of water, so that the volume changes observed were only a fraction of those to be expected with a dry gel. The density of the air-dry gelatin was 1·412. From the densities found by Lüdeking, the contractions in cc. have been calculated assuming the water alone to have decreased in volume.

	Specific volume	Volume in cc. of gelatin in 100 g. of gel	Contraction per cc. of water
Air dry gelatin	0·7082	—	—
10 g. gelatin + 90 g. H_2O	0·9354	7·08	0·040
25 g. gelatin + 75 g. H_2O	0·8810	17·70	0·063
50 g. gelatin + 50 g. H_2O	0·8052	35·41	0·098

These small contractions indicate that the first portions of water taken up are the more compressed. The maximum contraction corresponds to a density for the adsorbed water of about 1·11.

Information of more value is given by Rodewald's investigations on the contraction in total volume of starch and water during swelling [1897]. Rodewald used starch dried over sulphuric acid. This still contains some water, since drying *in vacuo* at 100° is necessary to drive off all the water [Dafert, 1887], but the contractions obtained should be much greater than with an air-dry gel. Assuming that the contraction is confined to the water, the volumes of 1 g. of water at each concentration have been calculated from Rodewald's results.

100 g. starch dried over H_2SO_4, containing water	Specific Volume	Vol. of adsorbed water in cc.	Vol. of 1 g. water in cc.	Vol. of 1 g. additional water, after subtracting vol. (=3·01 cc.) of first 9·8 g.
0	0·72585	—	—	—
1·17	0·72124	0·38	0·33	—
2·69	0·71319	0·78	0·29	—
5·02	0·70486	1·54	0·31	—
7·40	0·69881	2·67	0·36	—
9·70	0·69344	2·79	0·29	—
13·41	0·68727	6·60	0·49	0·91
14·95	0·68555	8·22	0·55	1·01
19·24	0·68111	9·51	0·49	0·68

It will be noted that up to about 9·8 per cent. of water, in addition to that already contained in the starch, the contraction of the adsorbed water is practically constant. Subtracting the volume of this 9·8 per cent. of water from the calculated volumes of adsorbed water at larger concentrations gives

practically no contraction for the additional water, except in the last case. From these figures it is possible to get a very rough idea of the pressures under which the water is imbibed.

The work of Worthington, Richards and Matthews, and others indicates that the product of the internal pressure, P, of a liquid and its compressibility, β, is approximately constant, *i.e.* $P\beta = C$. Amagat's figures [1893] show that this is not quite true for water. But, from the anomalous nature of water as a liquid, it is probable that the relation between its internal pressure and compressibility is not quite simple. Since the available data are so meagre and have not been derived from the study of dry gels, all that can be expected is to show that the theoretical considerations lead to values of the right order.

Assuming the relation $P\beta = C$, and taking $P_0 = 1{\cdot}1 \times 10^4$, and

$$\beta_0 = 5{\cdot}2 \times 10^{-5}, \text{ gives } C = 0{\cdot}572.$$

And since

$$\beta = -\frac{1}{V}\frac{dV}{dP}$$

$$\therefore \frac{dV}{V} = -0{\cdot}572\,\frac{dP}{P}$$

$$\text{or } \log_{10}\frac{V_0}{V_1} = 0{\cdot}572\,\log_{10}\frac{P_1}{P_0}.$$

Since the first 9·8 g. of water become 3·01 cc., the average pressure producing this contraction is given by

$$\log\frac{9{\cdot}8}{3{\cdot}01} = 0{\cdot}572\,\log\frac{P_1}{1{\cdot}1\times10^4}$$

which gives $P_1 = 8{\cdot}6 \times 10^4$.

From the last line in the table, the additional 9·44 g. of water adsorbed become 6·50 cc., from which $P_2 = 2{\cdot}1 \times 10^4$.

The heat evolved in the contractions should be given approximately by

$$Q = \frac{(P_1 - P_0)(V_0 - V_1)\times10^6}{42\times10^6}\text{ cal.}$$

$$\therefore\ Q_1 = 1{\cdot}2 \times 10^4 \text{ cal, and } Q_2 = 7 \times 10^2 \text{ cal}$$

which is negligible as compared with Q_1. This is the heat evolved by 100 g. of starch. The heat evolved per gram is therefore 120 cal.

Rodewald found about 30 cal. per g. for starch, which is only a quarter of this amount. There is, however, another change which takes place during swelling, in addition to the condensation of the water, and this causes absorption of heat. In accordance with Laplace's conception, the presence of a liquid will reduce the unbalanced attractions in the surface layer of the solid, in consequence of the attraction of the liquid molecules in the opposite sense. The surface tension of the solid will therefore be decreased, and

expansion of the surface layers will take place, with absorption of heat. The observed heat of imbibition is, therefore, less by this amount.

The heat of imbibition of gelatin was determined by Wiedemann and Lüdeking [1885]. Here again unfortunately air-dry substance was used, so that a value of only 5·7 cal. per g. was obtained. Before the employment of only air-dry substance was realised, the low value of the heat of swelling seemed a serious difficulty in working out the present theory. A determination of the heat of swelling of gelatin dried at 110° gave 32·96 cal. per g. as the mean of two experiments, or, allowing for the heat absorbed by the solation of gelatin by the water in the calorimeter, according to Wiedemann and Lüdeking's figures, the heat of swelling becomes 33·25 cal. This value is very similar to that obtained for starch.

The so-called "thermal anomaly" of gels was discovered by von Bjerkén [1891]. Arguing from a supposititious analogy with rubber, von Bjerkén expected to find that stretched gels would likewise contract on heating. From a number of experiments which were confined to gelatin gels, of which neither the quality, composition, nor form is recorded, the expectation appeared to be substantiated. No explanation of such a phenomenon is afforded by the theory considered in this paper. However, having regard to the tough skin which forms on the surface of gelatin gels, it seemed probable that such gels would contract in one direction when heated, owing to an approach to spherical shape under the influence of thermal expansion. This contraction would be dependent on the shape of the gel, and independent of any stretching force applied to the gel except indirectly as the result of a change induced in its shape. The contraction would also be affected by the variation in the elasticity of the outer skin with age or change of environment. The single case of a non-stretched gel recorded by von Bjerkén gave a very slight expansion in the unstretched state, and only a small contraction when stretched. The definite expansion is curious, since experiments show that expansion in the direction of greatest length is due to relaxation of the skin and proceeds without limit until the gel melts. This suggests that, in the unstretched state, the gel in question had its greatest dimension in some other direction than the one under observation.

Von Bjerkén himself records a noticeable small gradual expansion, which was superposed upon the contraction effect. Doubtless this was due to the gradual relaxation of the surface skin. It was therefore determined to reinvestigate the thermal behaviour of gelatin ribbons and cylinders, and the results of over seventy experiments completely fulfil expectations. The

gels showed either unlimited expansion, or contraction which varied with the age or previous history of the surface skin. Cutting or injuring the skin caused expansion instead of contraction.

No exact formula can be obtained to express the relation of the contraction to the form of the gel. An approximate one may be arrived at by supposing the volume, $V = \pi r^2 h$, of a cylinder of gel to increase, while the surface, $S = 2\pi rh + 2\pi r^2$, remains constant[1]. This gives

$$dS = 2\pi h dr + 2\pi r dh + 4\pi r dr = 0$$

or

$$dr = -\frac{r dh}{h+r}.$$

The change in volume,

$$\begin{aligned} dV &= 2\pi rh dr + \pi r^2 dh \\ &= \pi r^2 dh - \frac{2\pi r^2 h dh}{h+r} \\ &= -\frac{\pi r^2 (h-r)}{h+r} dh \\ \therefore \frac{1}{V} \cdot \frac{dV}{d\theta} &= -\frac{1}{h} \cdot \frac{h-r}{h+r} \cdot \frac{dh}{d\theta}. \end{aligned}$$

A cylinder is, however, not so convenient to work with as a ribbon, since there is danger that thermal equilibrium may not be reached during the rapid temperature changes required to prevent the gel melting. Only a still more approximate formula for a ribbon can be deduced. By writing $\rho = \frac{\sqrt{a+b}}{2}$, where a and b are the shorter dimensions of the ribbon, the equation becomes

$$\frac{1}{V} \cdot \frac{dV}{d\theta} = -\frac{1}{h} \cdot \frac{h-\rho}{h+\rho} \cdot \frac{dh}{d\theta}.$$

Since the surface skin of agar gels is very much softer than that of gelatin gels, it would be expected that the former would show no contraction on heating. This was found to be the case. It may therefore be regarded as established that the contraction in greatest length of gelatin gels when heated is due to the constricting effect of the surface skin as the gel expands in volume in accordance with its coefficient of thermal dilation, and is independent of any stretching force applied to the gel.

In so far as the phenomena exhibited by gels depend on the interrelation between the liquid and solid phases, they should be influenced by the well known lyotrope series of dissolved salts, which affect the surface tension and the affinity of the liquid and solid phases. The connection between adsorption of solvent and swelling, and the similar effect of the Hofmann series of anions on both was pointed out by Lillie [1907]. He suggested that osmotic pressure is due to adsorption of solvent by the dissolved particles, and showed

[1] Actually if the surface were inelastic the gel could not change in shape.

that the osmotic pressure of emulsoid sols is decreased by salts in the same order, $CNS < I < NO_3 < Br < Cl < SO_4 < CO_3$ as in the case of solutions. The diminished pressure would not appear to be due to increased aggregation of the sol particles, since the precipitating effect of the cations has not commenced at the concentrations employed, although anions hinder the precipitation in the opposite order [Pauli, 1913]. The same relations were found by Hatschek for the clearing of oil emulsions. The temperature of gelation should however be depressed in the order $CNS > I > \ldots > CO_3$ as was found by Levites [1908]. The change in the viscosity with time [Schröder, 1903] may be deduced in the same way as due to a diminution of the precipitating forces and the formation of fewer crystallisation centres, or sol particles. If the velocity of crystallisation is sufficiently reduced, gelation may be altogether prevented, as by the addition of the thiocyanate anion. The solubility of the colloid particles is temporarily increased thereby, their aggregation hindered and the sol stage prolonged. In a similar way the elasticity of gels is increased by salts which favour imbibition and *vice versa*. The double refraction of deformed sols shows the same lyotrope effect [Lieck, 1904].

4. Experimental.

The gelatin used in all, except the earliest, of these experiments was Nelson's "Crystal" leaf gelatin. It contained 15·41 per cent. of moisture, 1·66 per cent. of ash and only a trace of phosphate. The ash appeared to be due mostly to the presence of calcium bicarbonate in the gelatin.

A quantity of this gelatin was soaked for 6 days in running distilled water and quickly evaporated until the gel corresponded to 10 per cent. of the original gelatin. This gel gave no precipitate when treated with lime-water, or sodium phosphate solution, and only a trace of precipitate in the form of finely divided bands, with silver nitrate solution. It did not give the Liesegang phenomenon until citric acid had been added. Contrary to Liesegang's experience it was then unnecessary to add additional gelatose.

The gel so prepared was, however, quite unsuitable for the investigation of the thermal anomaly of gelatin, since the gelatin had almost completely lost its coherence, together with its property of forming a hard surface skin. It was impossible to mould a cylinder or ribbon which could be handled without breaking. This behaviour was identical with that of a sample of gel similarly prepared from another specimen of gelatin. The gelatin was further purified by two precipitations by pouring the aqueous sol into alcohol.

A number of ribbons made from this gelatin had also very soft surface skins and were equally fragile. From this it appears that the cohesion of gelatin is due to a water soluble substance, probably gelatose, which is removed by soaking. Consequently the leaf gelatin was used for the experiments in its original state. The agar gels were prepared directly from ordinary commercial shredded agar.

The so-called "Thermal anomaly" of gels.

The apparatus employed by v. Bjerkén for the observation of this property consisted of an apparently thick glass beaker, provided with a stirrer, and containing paraffin oil in which the ribbon of gel was mounted. The beaker was surrounded with an outer jar containing water, by changing which the temperature of the paraffin oil bath was regulated. Since the gelatin is heated to about 40°, which is much over its melting point, it is important that the temperature changes should be as rapid as possible. Consequently a modified form of apparatus was used. This comprised a thin glass tube 10 × 2 cm., with an overflow lip above, and having a side tube 1 cm. diam. at the base, where the main tube was constricted to 5 mm. diam. Through the constricted tube a glass rod passes freely, provided with a platinum hook above for the attachment of the gelatin ribbon. The space between the rod and the tube was closed by an outer sleeve of rubber tube. The rod was long enough for the hook to be passed to the top of the main tube when fitting the ribbon. It was then carefully drawn down, until the gel was in position, and securely clamped in place. By means of the side tube a rapid stream of liquid could be passed through the main tube from either of two thermostats at the temperatures selected for the experiment. These were usually that of the room and about 40°. The leading tubes were 1 cm. in diameter and, to avoid constriction, rubber connections and pinch cocks were employed. The side tube was fitted at right angles below with a waste tube, of the same diameter, for the rapid emptying of the main tube. The fluid found most convenient was liquid paraffin B.P. Observation showed that the change in length of the gel when immersed in water is inappreciable during the short time of the experiment, and this liquid was used on some occasions as a check. Water has also the advantages of greater heat capacity and mobility. When using liquid paraffin a small correction for the difference in temperature of the bath from that of the thermostat must be applied. With water there is no correction.

The gel cylinders or ribbons were moulded with metal plates embedded in the surfaces at the two ends. The plates were provided with holes through

which wires could be hooked for attachment. The lower end of the gel was hooked to the sliding glass rod, and the upper end was connected to a light lever and pointer, by means of which the position of the end of the gel could be estimated, with the aid of a lens, to within 0·001 mm. The weight of this lever caused a pull of only 6·5 g.

The preparation of the cylinders and ribbons was a matter of considerable difficulty, since it was essential to secure gels of which the surface was uninjured. Glass moulds, which were afterwards broken, were of no use. Moulds of silver foil formed round a glass tube and clamped down the side by a bull-dog clip, the edges of which had been filed square, seemed most convenient. The lower end of the tube so formed was closed by a split cork carrying the tongue of the circular plate carried above for the attachment of the gel, and was luted with paraffin wax if necessary. The upper plate was suspended by a pin through the perforation of the tongue, the cylinder filled with gelatin and allowed to set. The clamp was then removed and the cylinder weighed and slipped into a small test tube which was corked until required for use. The ribbons were moulded in troughs formed of extra stout tin foil, and kept in corked test tubes. The rate of loss of moisture from the gel was found to be fairly constant at about 0·6 per cent. in the time, about 20 hours, before measuring the expansion. From the time of taking out of the tubes it was impossible to control the loss of water. The compositions of the gels are therefore given as they were prepared, and their history recorded in each case. As a guide to their water content, the rate of loss of moisture by a sugar loaf of 10 per cent. air-dry gelatin weighing 15·147 g. exposed to the air under a raised inverted funnel was determined, see Fig. 2. To open the mould, the sheet metal was carefully peeled off the gel. Usually the surface of the gel was little damaged in the process, but it was never obtained quite perfect. Accordingly the gel was hung on a hook and painted all over with a hot gelatin sol of the same concentration. After drying for several hours in the air, the gel was carefully mounted in the apparatus and the cold liquid run through for about 30 seconds to insure temperature equilibrium. If the surface of the gel was even slightly injured in mounting, the gel subsequently gave no contraction. After 30 seconds the scale reading was taken, the cold liquid quickly run out, and a rapid current of hot liquid maintained until the maximum contraction had been recorded. This took about 15 seconds in liquid paraffin and half that time in water. The current of hot liquid was immediately changed for cold, before the gel melted, when the gel invariably expanded to beyond its original volume, the expansion continuing for some

time. This appeared to be due to the softening and gradual expansion of the outer skin by the hot liquid and the pull of the light weight of the lever.

From upwards of seventy experiments, every degree of effect was obtained from zero to a more or less definite maximum contraction, as well as

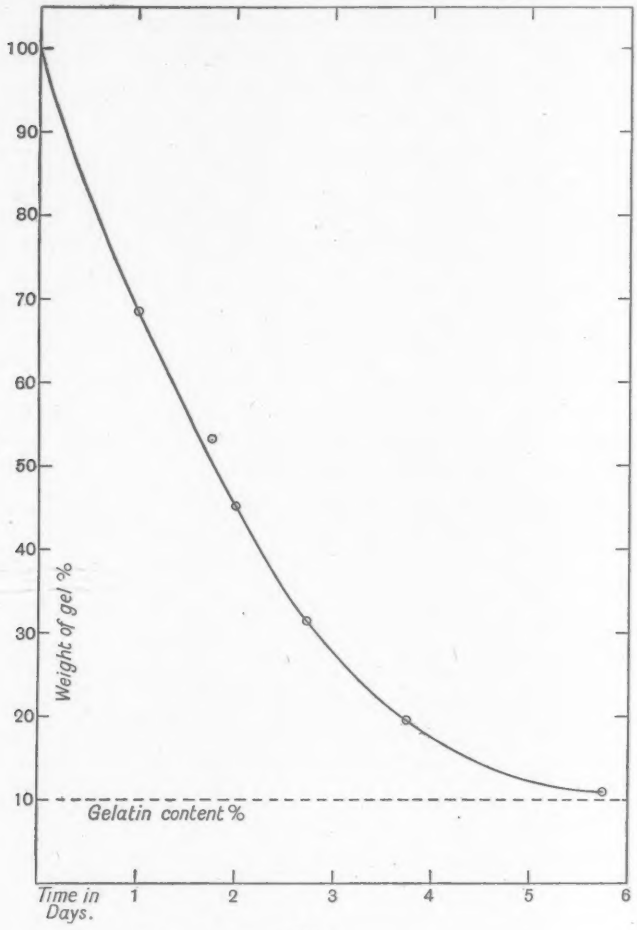

Fig. 2

indefinite expansion until melting occurred, according to the state of the outer skin of the gel. Usually very small contractions were observed on the first day, but after leaving the gel mounted in the empty apparatus over

night, about 16 hrs., a much greater effect was usually obtained. Keeping the gels in oil prevented any increase in contraction with time, whether before or after the first measurement. A second heating immediately following the first gave much less, or no contraction; a third consecutive heating invariably none. Keeping for 16–24 hrs. however usually restored or increased the effect. If the heating was continued too long, the outer skin melted before the interior of the gel and rapid expansion immediately occurred. That the contraction in length was due to an actual expansion in volume was proved by the facts that cylinders of less height than diameter increased in height on heating, and that gels confined in test-tubes, so that increase of diameter was prevented, also showed a linear expansion.

On account of its lack of adhesiveness, there was no difficulty in moulding cylinders of agar gel. These were prepared of 2·5 per cent. agar. They came out of the small glass tubes without any injury, but always showed immediate expansion in length when heated.

The results of a selection of the experiments are tabulated below. The specific linear contractions are given, as well as the calculated specific volume expansions, for comparison with von Bjerkén's data. No. 5 was a cylinder with height greater than its diameter. The times are measured from the moment of painting. The dimensions are given in cm.

The results appear to indicate, either that the actual thermal expansion of fresh gelatin gels is greater than that of the contained liquid, or that it becomes so with time. A number of experiments by various methods were made to determine this effect. Unexpected difficulties were encountered in obtaining concordant results, and when such had been obtained by one method, the values were found to differ greatly from an equally concordant set resulting from a different process. The question requires further study, and it is proposed to investigate the densities and coefficients of expansion of pure dry gelatin and agar and of a series of gels and sols of different concentration and age. This will also determine the total contraction of gel and water on swelling.

The Heat of Imbibition of Gelatin.

The heat of imbibition of air-dry sheet gelatin was found by Wiedemann and Lüdeking [1885] from three experiments as 6·2, 5·3 and 5·6 cal. giving a mean value of 5·7. The same observers obtained the value − 3·7 as the heat of solation of 1 g. of gelatin.

The gelatin for the present experiment was dried in the air oven at 110°

No.	Age	Concentration, etc.	Dimensions in cm.	Liquid in bath	$\frac{dL}{d\theta}$	$\frac{1}{h}\frac{dL}{d\theta}$	$\frac{1}{V}\cdot\frac{dV}{d\theta} = -\frac{1}{h}\frac{h-r}{h+r}\frac{dh}{d\theta}$
3	24 hrs.	10 % gelatin, old sample	$7{\cdot}5\times1{\cdot}3$	water	$-0{\cdot}0030$	$-0{\cdot}0_3 4$	$+0{\cdot}0_3 3$
4	24 ,, ...	,, ,, ,,	$7{\cdot}5\times1{\cdot}3$	,,	$-0{\cdot}0030$	$-0{\cdot}0_3 4$	$+0{\cdot}0_3 3$
5	3½ ,, ...	,, ,, ,,	$1{\cdot}0\times1{\cdot}25$	,,	Expanded $+0{\cdot}0_3 138$	$+0{\cdot}0_3 138$	$+0{\cdot}0_4 28$
13	3 ,, ...	,, ,, ,,	$5{\cdot}9\times0{\cdot}93$	,,	$-0{\cdot}0_3 188$	$-0{\cdot}0_3 32$	$+0{\cdot}0_3 2$
16	Reheated immediately	Same gel	—	,,	No contr.	—	—
17	16 hrs.	10 % Crystal gelatin ...	$4{\cdot}5\times0{\cdot}75$	liquid paraffin	$-0{\cdot}0_3 67$	$-0{\cdot}0_3 15$	$+0{\cdot}0_3 13$
18	Reheated	Same gel	—	,,	$-0{\cdot}0_3 49$	$-0{\cdot}0_3 11$	—
19	16 hrs.	10 % Crystal gelatin ...	$3{\cdot}9\times0{\cdot}65$	,,	$-0{\cdot}0_3 33$	$-0{\cdot}0_4 8$	$+0{\cdot}0_4 7$
20	After another 24 hrs. in app.	Same gel lengthened by hanging	$4{\cdot}1\times0{\cdot}55$	,,	$-0{\cdot}0_3 179$	$-0{\cdot}0_3 437$	$+0{\cdot}0_3 381$
22	16 hrs.	10 % Crystal gelatin ...	$4{\cdot}3\times0{\cdot}6$	,,	Expanded	—	—
23	24 hrs. longer in app.	Same gel	$4{\cdot}5\times0{\cdot}55$	,,	$-0{\cdot}0_2 296$	$-0{\cdot}0_3 658$	$+0{\cdot}0_3 578$
27	16 hrs. hung over water in covered beaker	10% Crystal gelatin ...	$6{\cdot}3\times1{\cdot}1\times0{\cdot}3$	,,	$-0{\cdot}0_3 22$	—	—
28	3½ hrs. later ...	Same gel	$6{\cdot}7\times1{\cdot}0\times2{\cdot}5$	,,	$-0{\cdot}0_2 249$	$-0{\cdot}0_3 372$	$+0{\cdot}0_3 291$
29	16 hrs.	10 % Crystal gelatin, surface injured	$4{\cdot}6\times0{\cdot}66$	,,	Expanded	—	—
30	21 hrs. longer in app.	Same gel	Unchanged	,,	$-0{\cdot}0_2 198$	$-0{\cdot}0_3 430$	$+0{\cdot}0_3 386$
31	3 hrs.	10 % Crystal gelatin ...	$5{\cdot}9\times1{\cdot}2\times0{\cdot}3$	,,	$-0{\cdot}0_3 93$	$-0{\cdot}0_3 157$	$+0{\cdot}0_3 144$
32	3 ,, ...	,, ,, ,,	$5{\cdot}1\times1{\cdot}2\times0{\cdot}33$	,,	Expanded	—	—
33	3 ,, ...	,, ,, ,,	$5{\cdot}0\times1{\cdot}1\times0{\cdot}3$	,,	$-0{\cdot}0_2 111$	$-0{\cdot}0_3 222$	$+0{\cdot}0_3 192$
34	Same gel 24 hrs. longer in app.	,, ,, ,,	$5{\cdot}3\times1{\cdot}0\times0{\cdot}26$	,,	$-0{\cdot}0_2 439$	$-0{\cdot}0_3 809$	$+0{\cdot}0_3 736$
36	24 hrs. in app. ...	,, ,, ,,	$6{\cdot}1\times1{\cdot}1\times0{\cdot}35$	,,	$-0{\cdot}0_2 108$	$-0{\cdot}0_3 178$	$+0{\cdot}0_3 161$
54	7 hrs.	12·5 % Crystal gelatin ...	$5{\cdot}3\times1{\cdot}25\times0{\cdot}35$	,,	$-0{\cdot}0_3 26$	$-0{\cdot}0_4 49$	—
56	7 ,, ...	,, ,, ,,	$5{\cdot}1\times1{\cdot}1\times0{\cdot}3$	,,	Expanded	—	—
57	Same gel 24 hrs. longer in app.	,, ,, ,,	$5{\cdot}2\times1{\cdot}1\times0{\cdot}3$	,,	$-0{\cdot}0_2 498$	$-0{\cdot}0_3 958$	$+0{\cdot}0_3 731$
58	24 hrs.	,, ,, ,,	$4{\cdot}9\times1{\cdot}0\times0{\cdot}3$	,,	$-0{\cdot}0_2 233$	$-0{\cdot}0_3 476$	$+0{\cdot}0_3 364$
68	5 ,, ...	15 % Crystal gelatin ...	$5{\cdot}7\times1{\cdot}3\times0{\cdot}2$	water	$-0{\cdot}0_2 242$	$-0{\cdot}0_3 428$	$+0{\cdot}0_3 326$
69	5 ,, ...	,, ,, ,,	$5{\cdot}9\times1{\cdot}3\times0{\cdot}3$	,,	$-0{\cdot}0_2 223$	$-0{\cdot}0_3 379$	$+0{\cdot}0_3 288$.
70	Same gel 24 hrs. longer in app.	,, ,, ,,	$5{\cdot}9\times1{\cdot}1\times0{\cdot}3$	,,	$-0{\cdot}0_2 668$	$-0{\cdot}0_2 1132$	$+0{\cdot}0_3 918$

On account of the approximate nature of the formulae and of the magnitude of the effect of small errors in measurement and of irregularities in form, on the calculated volumes of the gels, the figures are not of great intrinsic value.

for about 24 hours until constant in weight. The loss corresponded to 15·70 per cent. of moisture.

A small Dewar vacuum flask, holding about 150 cc., was used as calorimeter and was provided with a mechanical glass stirrer so that the rate of cooling was nearly constant. The temperatures were indicated by a Beckmann thermometer. The change of temperature by radiation per degree difference was only 0·0015° per 15 seconds. Practically the whole of the heat was evolved within about four minutes. The results were corrected by Regnault's method. Two determinations gave:

	(1)	(2)
Gelatin taken	3·942	2·083
Total water equivalent	147·841	139·845
Initial temperature of gelatin	21·2°	19·2°
Initial temperature of calorimeter	20·500°	18·486°
Final temperature	21·413°	18·977°
Rise in temperature	0·913°	0·491°
Corresponding heat value	132·368 cal.	68·664 cal.
Heat added with gelatin	1·242 „	0·656 „
Heat of swelling per g. of dry gelatin ...	33·264 „	32·649 „
Heat absorbed by solation of gelatin per g....	0·177 „	0·304 „
Corrected heat of imbibition	33·541 „	32·953 „
Mean	—	33·25 „

The actual heat of swelling is greater than the observed value, in consequence of the absorption of heat by the solation of some of the gelatin by the water in the calorimeter. This is proportional to the mass of water used. The exact amount solated during the time of the experiment is however unknown. An experiment showed that 100 g. of water are capable of taking up 0·114 g. of solid matter from sheet gelatin at 20·2° during the time of the experiment, which corresponds to 0·135 g. of air-dry gelatin. Using this value and Wiedemann and Lüdeking's figure for the heat of solation, the quantities of heat absorbed are 0·177 cal. and 0·304 cal. per gram respectively.

The values obtained do not agree very well. This may be due to the great influence of the previous thermal history of the gelatin upon its structure. It will be noticed that Wiedemann and Lüdeking's results showed a similar variation.

The writer wishes to express his indebtedness to Prof. J. C. Philip for the kind loan of the calorimetric apparatus.

SUMMARY.

The low diffusion constant of the natural emulsoids, by retarding the crystallisation of the hot sols, allows the accumulation of a large excess concentration, which, together with their large value of K in von Weimarn's formula, causes gelation on cooling. The small crystallisation velocity, due to this low diffusion constant, is also responsible for the permanency of the gels and the hysteresis of the sols, since it prevents subsequent development of larger crystals in gels and retards changes in the aggregation of sol particles.

The viscosity changes in the sols of the natural emulsoids, by heating, are seen to be in conformity with Einstein's formula, since thermal changes cause alterations in the numbers, as well as the size, of the crystallisation centres.

The application of von Weimarn's theory to the reversible sol-gel transformation implies that gels should comprise two phases: an ultramicroscopic solid phase, bathed in a liquid dispersion medium, from which it has crystallised, and which it retains partly by molecular and partly by capillary forces.

Consideration of the conditions of occurrence of spherites shows that complex organic substances and such as are highly aggregated in solution tend to crystallise in this form, and this leads to the supposition that the ultimate solid particles of gels are spherites. This is in conformity with the ultramicroscopic appearance, and receives further support from the fact that, by suitable means, gelatin can be made to assume the form of microscopic spheres.

The specific surface of gels is very large, and consequently the volume of liquid adsorbed at the liquid-solid boundary surfaces is appreciable. This liquid is under an internal pressure intermediate between those of the solid and of the liquid. This intermediate value of the internal pressure is much greater than that of the free liquid and conditions certain peculiarities in the properties of gels.

The theory forms a basis for the coordination of the properties of gels, and those predicted for such disperse systems are in accordance with the phenomena exhibited by gels. The experimental data at present available are insufficient for a complete mathematical discussion of the theory. Where the previous records are at variance with the theory, the data have been found to be in error.

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XXX. THE EFFECT OF ACIDS ON THE GROWTH OF *BACILLUS COLI.*

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(A report to the Medical Research Committee.)

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The original proposition advanced by Michaelis and Marcora [1912] stated that the final acid reaction of *B. coli* constituted a "physiological constant" of the organism, since cultures in lactose bouillon ceased activity at a hydrogen ion concentration [H·] of $1 \times 10^{-5} N$. The results of their experiments were soon challenged by Brünn [1913] who showed that an acid reaction of $P_H = 5{\cdot}0$ permitted, but that $P_H = 4{\cdot}7$ inhibited growth of *B. coli*.

More recently Clark and Lubs [1915, 1, 2 and 3] have examined the conditions affecting the growth of *B. coli* of the low-gas-rated group, and they find a certain connection between the type of colon bacillus used, and the final acid reaction it produces.

Clark [1915] determined the final [H·] attained by cultures of *B. coli* and although he agrees with the hypothesis of Michaelis and Marcora, he presents the following additional facts:

(*a*) In a medium containing 1 per cent. Witte peptone and 1 per cent. dextrose the final acid reactions attained are:

'Human' Coli $P_H = 4{\cdot}82$
'Bovine' ,, $P_H = 4{\cdot}7$–$4{\cdot}8$
'Grain' ,, $P_H = 5{\cdot}01$.

He suggests that these reactions may be 'specific' for the respective varieties of *B. coli*, and that they may be of value for diagnostic purposes.

(*b*) A higher degree of acidity is produced in dextrose than in lactose media; *e.g.*, the same strain of *B. coli* produced in 1 per cent. Witte peptone + 1 per cent. dextrose a final reaction of $P_H = 4{\cdot}26$ while in 1 per cent. Witte peptone + 1 per cent. lactose only $P_H = 4{\cdot}56$ was attained.

(*c*) In the more highly buffered media lower $H^{\cdot}$ concentrations are produced.

In one series of his experiments a very significant result is obtained, viz. that the addition of various amounts of sterile lactic acid to the Witte peptone-dextrose medium before inoculation causes considerable variations of the final acid reaction attained by the cultures of *B. coli* in the acidified media.

These facts seem to cast considerable doubt upon the validity of the 'physiological constant' hypothesis, and the recent researches of Wolf and Harris [1917] on the behaviour of *B. sporogenes* and *B. perfringens* in acid media indicate the desirability of further investigation of the final $[H^{\cdot}]$ reached by cultures of *B. coli*.

Again, Cole and Onslow [1915] claim to be able to differentiate the organisms of the *B. typhosus* group by the velocity with which they attain a certain degree of acidity. Wolf and Harris [1917] state, however, that in the case of *B. sporogenes* and *B. perfringens* this velocity is affected by the initial $[H^{\cdot}]$ of the medium.

It was thought desirable, therefore, to undertake further examination of the behaviour of *B. coli* in different acid media. Several important questions at once suggest themselves, *e.g.*

(*a*) Do various strains of *B. coli* behave similarly when subjected to similar conditions?

(*b*) Does the nature and degree of acidity of the original medium affect the final reaction of the culture of *B. coli* grown in it?

(*c*) What is the mechanism of the action of 'Grain' Coli?

The present paper embodies results obtained in working on questions (*a*) and (*b*), and it is hoped to present additional results, together with a consideration of (*c*) in a later paper.

The main points now investigated are:

(i) Do different strains of the same variety of *B. coli* (*e.g.* human or bovine) react in the same way when exposed to similar conditions?

(ii) Are there any differences between the behaviour of 'human' and 'bovine' coli, such as may be made use of for diagnostic purposes?

(iii) Is the same strain of *B. coli* differently affected by different media?

I. Material and experimental methods.

Material.

The strains of *B. coli* used in these experiments were prepared as follows. Smears of human and bovine faeces were inoculated into a medium containing 2 per cent. glucose, 2 per cent. peptone water; and at the end of 24 hours MacConkey broth was inoculated from the growths so obtained. At the end of a further 24 hours the culture produced in the MacConkey broth was used for the inoculation of fresh MacConkey broth and from this, at the end of a further 24 hours, inoculation of agar slopes was effected. The strains thus produced were subjected to microscopic examination, single colonies were transferred to new agar slopes and from the daughter colonies developed thereon the pure strains used in the experiments were obtained. In addition to a large number of strains of human and bovine *B. coli* thus prepared a pure strain of *B. coli*, referred to in the paper as *B. coli oxf.* was obtained from Dr A. D. Gardner of the Oxford University Pathological (Standards) Laboratory, and this was used in the preliminary experiment for comparison of the different strains of human coli.

The strains of *B. coli* specially prepared for the experiments were lettered H_1, H_2, H_3 etc. (human) and B_1, B_2, B_3 etc. (bovine).

Media.

Two media were used:

(*a*) 2 per cent. glucose + 2 per cent. peptone water, prepared by the tryptic digestion of caseinogen (Lait Proto A) as described by Cole and Onslow, from which the tubes of acidified media were prepared by a method similar to that of Wolf and Harris.

(*b*) Glucose-phthalate mixture.

This was prepared as follows:

8·8 g. of anhydrous disodium monohydrogen phosphate
2·0 g. of potassium acid phthalate (recrystallised)
1·0 g. of aspartic acid
4·0 g. glucose

were dissolved in distilled water, the volume of the solution being made up to 800 cc.

This solution was then brought to the required concentration by taking, for the preparation of each 100 cc. of acid medium, 50 cc. of phthalate solution; x cc. of $N/5$ acid and $(50 - x)$ cc. of distilled water.

This glucose-phthalate medium was selected for use in these experiments because it had been found by Clark and Lubs to be a medium in which

B. coli can be grown with considerable facility, and it was also known to have a considerable 'buffer' effect: this being more marked than is the case with the glucose-peptone medium. *It was found that the gas production due to the activity of* B. coli *was much less in glucose phthalate than in glucose peptone.*

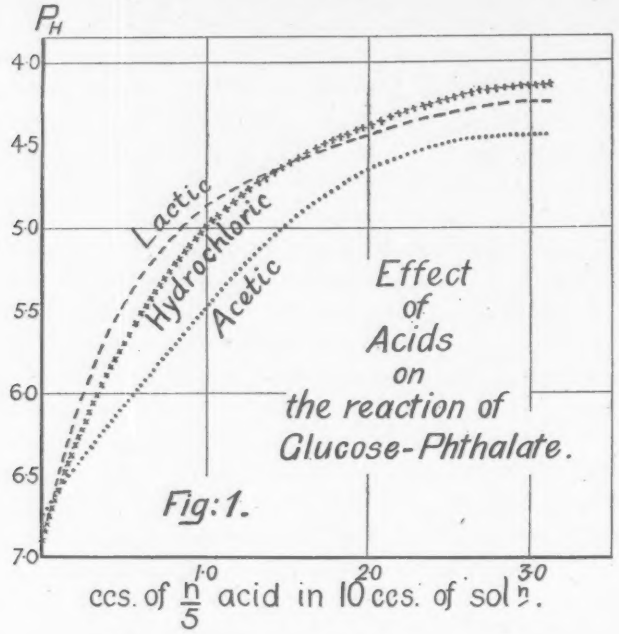
Experimental Methods.

For each series of experiments A, B, C, D, etc., a number of sets of tubes, 1, 2, 3, 4, etc., were prepared, each tube containing 10 cc. of a solution containing medium, acid, and water. The set of tubes containing the least amount of acid was numbered 1, the next more acid set 2,—the most acid set of tubes in each series receiving the highest number. In general, each set consisted of eight tubes of the same mixture, and each series comprised eight or ten sets of tubes. Into each tube of medium a sterilised Durham tube, for the collection of evolved gas, was introduced. The tubed media were sterilised at 100° for one hour per diem on each of three successive days. One tube of each set was used for the determination of the initial reaction of the sterilised medium. The reaction was determined at about 20°, and corrected to 20° when necessary. The H-electrode was used, and the P_H of the tubed medium was calculated from the E.M.F. so determined. The values of P_H were used in constructing the titration curves for the known media and acids employed.

The amounts of acid added to the media were so regulated as to produce a number of mixtures having P_H ranging from 4·0 to 7·0.

For each acid and medium a titration curve was constructed, in which the number of cc. of $N/5$ acid present in 10 cc. of the solution was plotted against the absolute acid reaction (P_H) produced. These curves showed that in order to produce a given acid reaction (P_H) in a medium, it was necessary to add to a given volume of it, a smaller mass of a highly dissociated acid (*e.g.* hydrochloric acid) than of a less dissociated acid (*e.g.* acetic acid).

The addition of the less dissociated acids soon produces an acid reaction (P_H) at which subsequent considerable additions of acid result in little change of reaction. In the more highly buffered media this point is more quickly attained. There does not appear to be any marked 'specificity' of action due to the *anion* groups of the various acids upon the media. Titration curves showing the effect of different acids upon 2 per cent. glucose peptone have been given by Wolf and Harris and as similar curves were obtained in the present experiments it was thought unnecessary to reproduce them. As the curve for glucose phthalate is not known it is shown in Fig. 1.



RESULTS OF INOCULATION EXPERIMENTS.

In the data given below strains H_1, H_2, etc. are *B. coli* obtained from human faeces; B_1, B_2, etc. prepared from bovine faeces, and "final P_H" is the lowest value recorded during a period (unless otherwise stated) of 168 hours.

+ indicates that fermentation occurred in the tube so marked; − means failure to ferment.

TABLE I. *Medium, 2 per cent. glucose peptone. Acid, hydrochloric.*

	Series Z_1 Inoculated with H_4 18 hrs. old			*Series Z_2* Inoculated with B_4 18 hrs. old			
Tube	Result	Initial P_H reaction	Final P_H reaction	Tube	Result	Initial P_H reaction	Final P_H reaction
1	+	6·66	4·65	1	+	6·66	4·64
2	+	6·23	4·54	2	+	6·23	4·50
3	+	5·73	4·45	3	+	5·73	4·46
4	+	5·42	4·37	4	+	5·42	4·43
5	+	4·95	4·33	5	+	4·95	4·42
6	+	4·79	4·30	6	+	4·79	4·35
7	+	4·64	4·29	7	+	4·64	4·30
8	+	4·45	4·29	8	+	4·45	4·31

(In both of these series all the tubes fermented, the most acid reactions obtained being: Series Z_1, Tubes 7 and 8 P_H=4·29, and Series Z_2, Tube 7 P_H=4·30.)

Series Y_1

Inoculated with H_1 18 hrs. old

Tube	Initial P_H reaction	Result	Final P_H reaction
1	5·89	+	4·50
2	5·24	+	4·40
3	4·91	+	4·38
4	4·62	+	4·30
5	4·02	–	—
6	3·96	–	—
7	3·53	–	—

Series Y_2

Inoculated with B_2 18 hrs. old

Result	Final P_H reaction
+	4·53
+	4·41
+	4·39
+	4·30
–	—
–	—
–	—

The tubes of Series Y_1 and Y_2 were examined 72 hours after inoculation.

The most acid tubes to ferment were those numbered 4 in each series, both of which had an initial reaction of $P_H = 4·62$ and reached a final reaction of $P_H = 4·30$. The next tube, No. 5 in each series, with initial reaction of $P_H = 4·02$ failed to ferment.

Series X_1

Inoculated with H_1 16 hrs. old

Tube	Initial P_H reaction	Result	Final P_H reaction
1	5·87	+	4·47
2	5·27	+	4·35
3	4·65	+	4·30
4	4·34	–	—
5	4·16	–	—
6	4·03	–	—
7	3·77	–	—
8	3·70	–	—

Series X_2

Inoculated with B_2 16 hrs. old

Result	Final P_H reaction
+	4·50
+	4·34
+	4·295
–	—
–	—
–	—
–	—
–	—

The tubes were examined 90 hours after inoculation.

In series X_1 the most acid tube to ferment was No. 3 with reaction $P_H = 4·65$, the acidity of which rose to $P_H = 4·30$. In series X_2 a similar result was obtained and a final value $P_H = 4·295$ was reached. It is noteworthy that in both series Tube No. 4 with an initial $P_H = 4·34$ failed to ferment.

Series O

Inoculated with *B. coli oxf.* 16 hrs. old

Tube	Initial P_H reaction	Result	Final P_H reaction
1	6·63	+	4·79
2	6·06	+	4·70
3	5·80	+	4·66
4	5·18	+	4·54
5	4·93	+	4·50
6	4·62	+	4·30
7	4·34	–	—
8	4·21	–	—

The most acid tube to ferment was No. 6 with initial reaction of $P_H = 4{\cdot}62$ and final reaction of $P_H = 4{\cdot}30$. It must be noted, however, that the next tube, No. 7, which failed to ferment had an initial reaction of $P_H = 4{\cdot}34$ which is less acid than the final value reached by the fermented No. 6.

The existence of this phenomenon in the case of *B. sporogenes* and *B. perfringens* has already been recorded by Wolf and Harris.

	Series S_1			*Series* S_2	
	Inoculated with H_s 18 hrs. old			Inoculated with B_s 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	7·14	+	4·53	+	4·53
2	5·17	+	4·39	+	4·45
3	4·52	+	4·30	+	4·34
4	4·29	+	4·26?	–	—
5	4·00	–	—	–	—
6	3·695	–	—	–	—
7	3·525	–	—	–	—
8	3·37	–	—	–	—

In Series S_1 the most acid tube certainly to ferment was No. 3, with initial $P_H = 4{\cdot}52$ which rose to $P_H = 4{\cdot}30$. Tube No. 4 with initial acid reaction $P_H = 4{\cdot}29$ rose to $P_H = 4{\cdot}26$ although the usual signs of fermentation were absent. Tube No. 5 with $P_H = 4{\cdot}00$ failed to ferment.

In Series S_2 the most acid tube to ferment was No. 3 with an initial acid reaction $P_H = 4{\cdot}52$ which rose to $P_H = 4{\cdot}34$. Tube No. 4 with $P_H = 4{\cdot}29$ failed to ferment.

TABLE II. *Medium, 2 per cent. glucose peptone. Acid, lactic.*

	Series W_1			*Series* W_2	
	Inoculated with H_s 16 hrs. old			Inoculated with B_s 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·46	+	4·60	+	4·61
2	5·46	+	4·48	+	4·47
3	4·53	+	4·47	+	4·46
4	4·39	–	—	–	—
5	4·15	–	—	–	—
6	3·98	–	—	–	—
7	3·83	–	—	–	—
8	3·73	–	—	–	—

In Series W_1 the most acid tube to ferment was No. 3 with acid reaction $P_H = 4{\cdot}53$ which rose to $P_H = 4{\cdot}47$. The next tube, No. 4, with $P_H = 4{\cdot}39$ failed to ferment.

In Series W_2 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4{\cdot}53$, which rose to $P_H = 4{\cdot}46$. The next tube, No. 4, with $P_H = 4{\cdot}39$ failed to ferment.

	Series T_1			*Series T_2*	
	Inoculated with H_s 18 hrs. old			Inoculated with B_s 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·90	+	4·69	+	4·70
2	4·88	+	4·61	+	4·60
3	4·62	+	4·57	+	4·56
4	4·44	–	—	–	—
5	4·275	–	—	–	—
6	4·08	–	—	–	—
7	3·93	–	—	–	—
8	3·70	–	—	–	—

In Series T_1 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4{\cdot}62$ which rose to $P_H = 4{\cdot}57$. The next tube, No. 4, with $P_H = 4{\cdot}44$ failed to ferment.

In Series T_2 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4{\cdot}62$ which rose to $P_H = 4{\cdot}56$. The next tube, No. 4, with $P_H = 4{\cdot}44$ failed to ferment.

TABLE III. *Medium, 2 per cent. glucose peptone. Acid, acetic.*

	Series V_1			*Series V_2*	
	Inoculated with H_s 16 hrs. old			Inoculated with B_s 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	7·14	+	4·52	+	4·54
2	5·39	+	4·60	+	4·61
3	4·83	+	4·77	+	4·79
4	4·60	–	—	–	—
5	4·46	–	—	–	—
6	4·41	–	—	–	—
7	4·35	–	—	–	—
8	4·28	–	—	–	—

In Series V_1 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4{\cdot}83$ which rose to $P_H = 4{\cdot}77$. The next tube, No. 4, with $P_H = 4{\cdot}60$ failed to ferment except in one doubtful case in which the P_H rose to 4·58.

In Series V_2 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4{\cdot}83$ which rose to $P_H = 4{\cdot}79$. The next tube, No. 4, with $P_H = 4{\cdot}60$ failed to ferment.

TABLE IV. *Medium, glucose phthalate. Acid, hydrochloric.*

	Series A_1			*Series* A_2	
	Inoculated with H_4 18 hrs. old			Inoculated with B_5 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·90	+	5·73	+	5·76
2	6·71	+	5·35	+	5·38
3	6·45	+	5·06	+	5·06
4	6·25	+	4·93	+	4·94
5	5·87	+	4·83	+	4·83
6	5·66	+	4·77	+	4·76
7	5·10	+	4·72	+	4·71
8	4·40	−	—	−	—

In Series A_1 the most acid tube to ferment was No. 7 with acid reaction $P_H = 5{\cdot}10$ which rose to $P_H = 4{\cdot}72$. The next tube, No. 8, with $P_H = 4{\cdot}40$ failed to ferment.

In Series A_2 the most acid tube to ferment was No. 7 with acid reaction $P_H = 5{\cdot}10$ which rose to $P_H = 4{\cdot}71$. The next tube, No. 8, with $P_H = 4{\cdot}40$ failed to ferment.

Series N

	Inoculated with *B. coli oxf.* 16 hrs. old		
Tube	Initial P_H reaction	Result	Final P_H reaction
1	6·69	+	5·21
2	6·56	+	5·20
3	6·44	+	5·18
4	6·15	+	5·12
5	5·89	+	5·05
6	5·70	+	4·94
7	5·37	+	4·91
8	4·85	+	4·78
9	4·31	−	—
10	3·70	−	—
11	3·31	−	—

The most acid tube to ferment was No. 8, with acid reaction $P_H = 4{\cdot}85$ which rose to $P_H = 4{\cdot}78$. The next tube, No. 9, with $P_H = 4{\cdot}31$ failed to ferment.

	Series C_1			*Series C_2*	
	Inoculated with H_4 18 hrs. old			Inoculated with B_4 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	7·11	+	5·60	+	5·43
2	6·80	+	5·11?	+	4·99?
3	6·69	+	4·99?	+	4·96?
4	6·52	+	5·05	+	5·04
5	6·27	+	5·01	+	5·01
6	6·13	+	4·93	+	4·96
7	5·95	+	4·94	+	4·88
8	5·77	+	4·89	+	4·91
9	5·01	+	4·75	+	4·76
10	4·96	+	4·68	+	4·75
11	4·41	–	—	–	—

In Series C_1 the most acid tube to ferment was No. 10, with acid reaction $P_H = 4·96$ which rose to 4·68. The next tube, No. 11, with $P_H = 4·41$ failed to ferment.

In Series C_2 the most acid tube to ferment was No. 10, with initial reaction $P_H = 4·96$ which rose to 4·75. The next tube, No. 11, with $P_H = 4·41$ failed to ferment.

TABLE V. *Medium, glucose phthalate. Acid, lactic.*

	Series K_1			*Series K_2*	
	Inoculated with H_7 16 hrs. old			Inoculated with B_7 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·90	+	5·75	+	5·75
2	6·00	+	5·02	+	5·05
3	5·55	+	4·94	+	4·95
4	5·20	+	4·82	+	4·85
5	5·10	+	4·78	+	4·76
6	4·99	+	4·78	+	4·79
7	4·85	+	4·76	+	4·75
8	4·71	–	—	–	—
9	4·55	–	—	–	—
10	4·44	–	—	–	—
11	4·26	–	—	–	—

In Series K_1 the most acid tube to ferment was No. 7, with an acid reaction of $P_H = 4·85$ which rose to $P_H = 4·76$. The next tube, No. 8, with $P_H = 4·71$ did not ferment.

In Series K_2 the most acid tube to ferment was No. 7, with an acid reaction of $P_H = 4·85$ which rose to $P_H = 4·75$. The next tube, No. 8, with $P_H = 4·71$ failed to ferment.

TABLE VI. *Medium, glucose phthalate. Acid, acetic.*

	Series D_1			*Series D_2*	
	Inoculated with H_8 16 hrs. old			Inoculated with B_8 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·85	+	5·13	+	
2	6·06	+	5·16	+	5·18
3	5·56	+	5·20	+	5·25
4	4·98	+	4·93	+	4·90
5	4·78	–	—	–	—
6	4·60	–	—	–	—
7	4·50	–	—	–	—

In Series D_1 the most acid tube to ferment was No. 4, with initial acid reaction $P_H = 4·98$ which rose to 4·93. In one tube of No. 5 (initial $P_H = 4·78$) the acid reaction rose to $P_H = 4·74$ but other signs of fermentation were absent. The next tube, No. 6, with $P_H = 4·60$ failed to ferment.

In Series D_2 the most acid tube to ferment was No. 4, with acid reaction $P_H = 4·98$ which rose to $P_H = 4·90$. The next tube, No. 5, with $P_H = 4·78$ failed to ferment.

TABLE VII. *Values of final acid reaction (P_H) of different strains of* B. coli *in different media and in the presence of different acids.*

		"Human" Coli			"Bovine" Coli		
Medium	Acid	Series	Strain	Final P_H	Series	Strain	Final P_H
2 % glucose peptone	Hydrochloric	O	Oxf.	4·30	—	—	—
		Z_1	H_4	4·29	Z_2	B_4	4·30
		Y_1	H_1	4·30	Y_2	B_2	4·30
		X_1	H_1	4·30	X_2	B_2	4·29
		S_1	H_8	4·30 (4·26?)	S_2	B_8	4·34
		Average value P_H = 4·30			Average value P_H = 4·31		
2 % glucose peptone	Lactic ...	W_1	H_3	4·47	W_2	B_4	4·46
		T_1	H_8	4·57	T_2	B_8	4·56
		Average value P_H = 4·52			Average value P_H = 4·51		
2 % glucose peptone	Acetic ...	V_1	H_8	4·77	V_2	B_8	4·79
Glucose phthalate	Hydrochloric	A_1	H_4	4·72	A_2	B_5	4·71
		A_3	H_4	4·72	—	—	—
		N	Oxf.	4·78	—	—	—
		C_1	H_4	4·68	C_2	B_4	4·75
		Average value P_H = 4·71			Average value P_H = 4·73		
Glucose phthalate	Lactic ...	K_1	H_7	4·76	K_2	B_7	4·75
Glucose phthalate	Acetic ...	D_1	H_8	4·88 (4·74?)	D_2	B_8	4·90 (4·82?)

Reaction resultants.

From the values shown in Tables I to VI curves giving the reaction resultants of the various strains of *B. coli* in 2 per cent. glucose peptone, and the glucose phthalate mixture were drawn by the method of Wolf and Harris [1917]. These show the behaviour of the organisms in these media in the presence of hydrochloric, lactic, and acetic acids, the initial acid reactions (P_H) being plotted against those finally produced (Figs. 2 ff.).

CONSIDERATION OF EXPERIMENTAL DATA.

It is now possible to suggest some conclusions relative to the questions originally proposed for investigation.

1. A comparison of the values of the final acid reactions shown in Tables I to VI indicates no specificity of reaction of the different strains of *B. coli*. This may be illustrated by the following examples:

(*a*) H_4 caused the acid reaction of glucose peptone and hydrochloric acid to rise from $P_H = 4{\cdot}64$ to 4·29, while H_1 caused a rise of P_H from 4·64 to 4·30, and *Oxf.* produced a rise of P_H from 4·62 to 4·30.

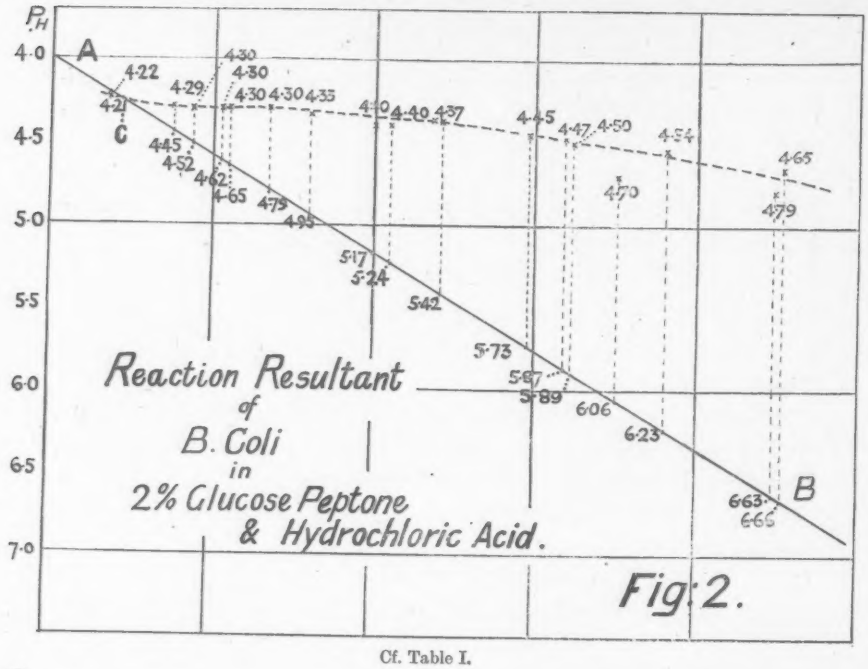
When glucose phthalate was the medium used, the results support the same conclusion, for example, *Oxf.* caused the reaction of the medium to rise from $P_H = 5{\cdot}95$ to 4·94, while H_4 caused a rise from $P_H = 5{\cdot}87$ to 4·83.

It may be concluded, therefore, that the different strains of the same variety of *B. coli* react in the same way when exposed to similar conditions.

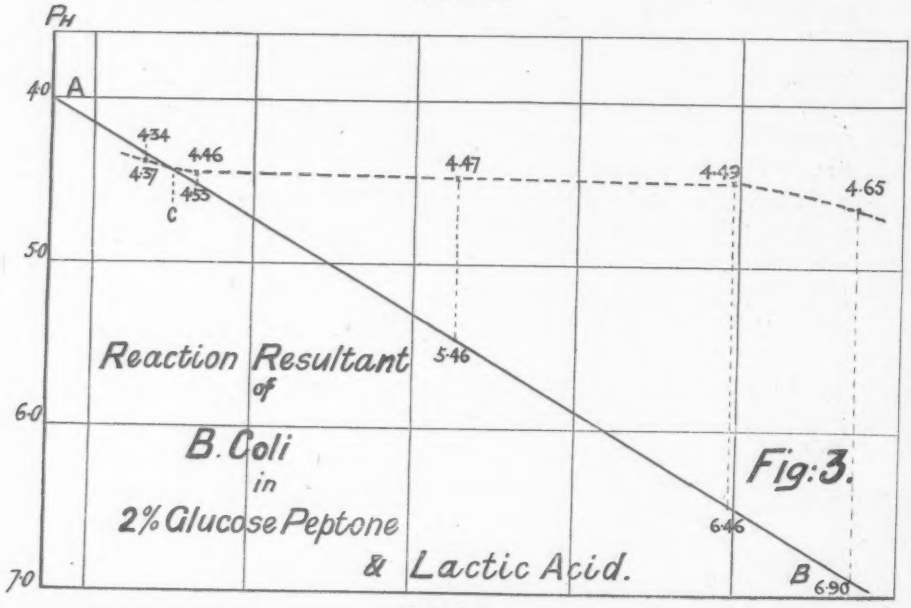
2. In no single case during the whole series of experiments, did any appreciable difference develop between the behaviour of "human" and "bovine" strains of *B. coli* (cf. Table VII).

It may be concluded therefore that the existence of differences between the behaviour of "human" and "bovine" coli such as may be of diagnostic value has not been demonstrated. In many of the experiments the two types of *B. coli* examined although fermenting under identical conditions behave identically at every stage of the experiment.

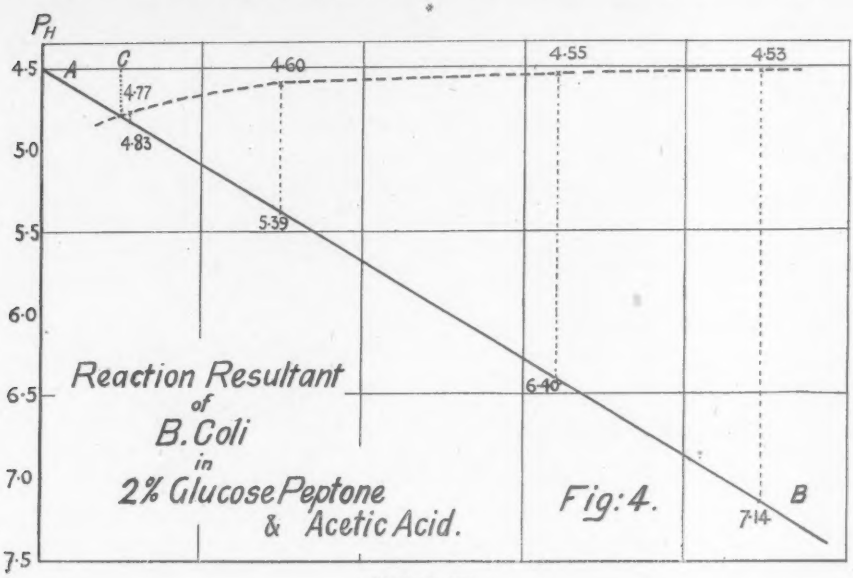
3. The experimental data lead to the conclusion that the same strain of *B. coli* is differently affected when allowed to ferment in different media. This is true both as to the final acid reaction attained in the presence of a given acid, and as to the rapidity with which this final reaction is reached. In Table I, Series Z_1, and Table IV, Series A_1, may be seen the results obtained by the fermentation of strain H_4 in glucose peptone and hydrochloric acid, and glucose phthalate and hydrochloric acid, respectively. When media to



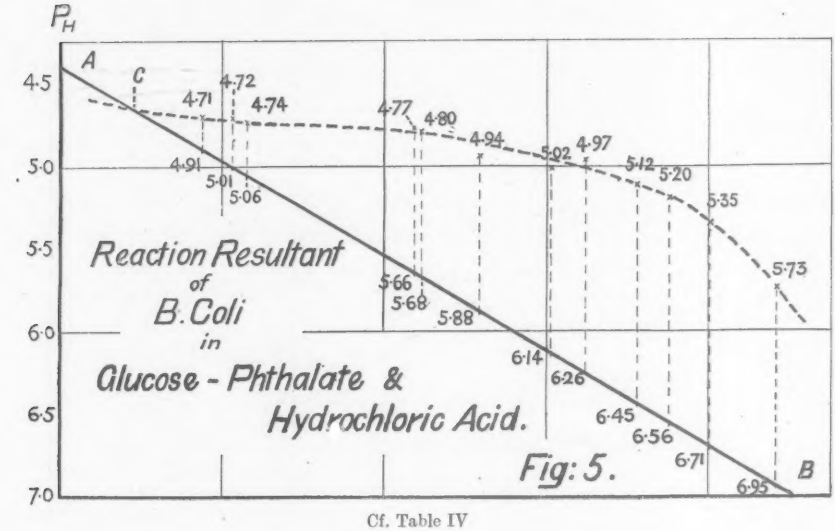
Cf. Table I.



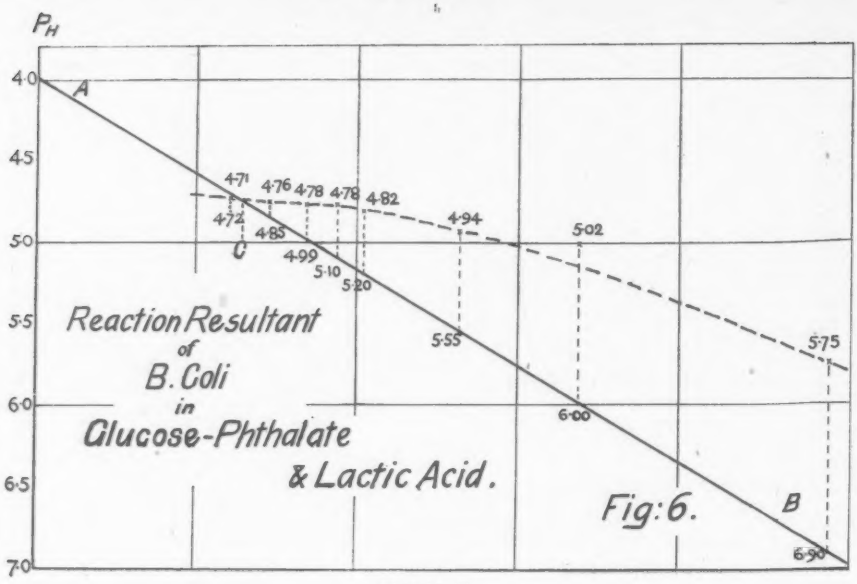
Cf. Table II.



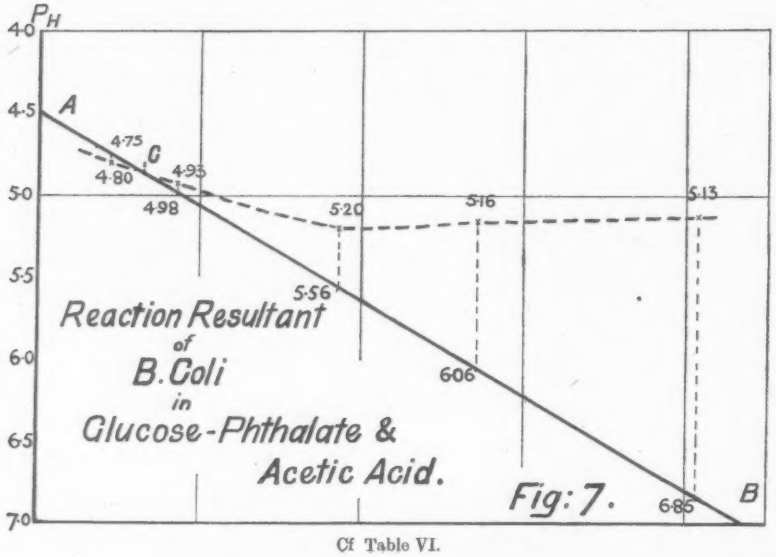
Cf. Table III.



Cf. Table IV



Cf. Table V.



Cf Table VI.

which no acid has been added are considered, it is evident that any given strain of *B. coli* causes a higher final acid reaction in peptone than in phthalate of similar initial reaction: *e.g.* H_4 fermenting in peptone to which no acid has been added, causes a rise of P_H from 6·66 to 4·65, while it produces in phthalate of initial $P_H = 6·91$ a final reaction of $P_H = 5·73$.

All the available evidence therefore leads to the supposition that *B. coli* ferments more strongly in glucose peptone than in glucose phthalate, whether the media be devoid of acid, or whether equivalent amounts of the same acid have been added to each.

4. An examination of Tables I to VI and of the curves in Figs. 2 to 7 (which represent the reaction resultants in the different media, and in the presence of various acids), proves conclusively that the final acid reaction produced by the fermentation of *B. coli* is by no means a "physiological constant." It cannot be doubted that the final reaction reached in any given experiment is dependent upon the initial reaction of the medium in which the organism is allowed to ferment. In Figs. 2 to 7 the forms of the curves obtained are evidently dependent upon the nature of the acid added to the medium, and also to a less extent upon the nature of the medium itself. The exact value of the acid reaction at which fermentation is completely inhibited is more conveniently discussed in connection with the next question.

5. It is evident that for any given medium there is a certain definite acid reaction at which the growth of *B. coli* is completely inhibited. It is possible to define this "critical point" within very narrow limits. In the figures of the reaction curves this point is represented by C, the point at which the curve representing the final reactions obtained in the medium cuts the axis along which the initial concentrations are plotted. The critical value may also be approximately taken in any given case as the mean of the values obtained for the final acid reactions and the lowest acid reaction of the medium which completely inhibits fermentation. These values are shown in the following table:

TABLE VIII.

Medium	Acid	Most acid reaction permitting growth	Least acid reaction inhibiting growth	Mean critical point (Calc.)	Critical point (Curve)
Glucose peptone	Hydrochloric	4·30	4·25	4·27	4·25
	Lactic	4·52	4·42	4·47	4·43
	Acetic	4·77	4·60	4·68	4·78
Glucose phthalate	Hydrochloric	4·71	4·38	4·55	4·67
	Lactic	4·76	4·71	4·73	4·74
	Acetic	4·88	4·78	4·83	4·85

It will be seen that columns 5 and 6 of the above Table define, within very narrow limits, the critical point for *B. coli* fermenting in the given media and acids.

Although all strains of a particular variety, and both the "human" and "bovine" varieties of *B. coli* appear to react similarly in the presence of a given acid and medium it is evident that, quite apart from the influence upon the acid reaction exerted by the particular medium, there is a small but definite result due to the specificity of action of the added acid. The titration curves of the different acids in any given medium reveal, as has already been noted, but little specificity of action; the final reactions obtained appear to be a measure of the true acidity produced by the acid. When the reaction resultants are examined a much greater specific action of the acid is visible.

The results obtained in the foregoing experiments suggest that in any given medium "a strong" or highly dissociated acid (*e.g.* hydrochloric or lactic) permits a higher final acid reaction before complete inhibition of fermentation takes place. When however the mass of acid introduced is considered, it is found that the strong acids are more strongly inhibitory than is the "weaker" or less dissociated acetic acid, *e.g.* in glucose peptone the addition of an amount of hydrochloric acid sufficient to produce an acidity equivalent to 0·0180 *N* completely inhibits growth, the final acid reaction reached being $P_H = 4{\cdot}30$ while the acid constitutes 0·0657 per cent. of the mass of the solution. With lactic acid growth ceases at $P_H = 4{\cdot}47$ when the acidity is 0·0180 *N* and the acid constitutes 0·1637 per cent. of the mass of the solution. With acetic acid although the final acid reaction at which fermentation is inhibited is only $P_H = 4{\cdot}77$, the acid added to the medium is equivalent to an acidity of 0·0240 *N* and is 0·1440 per cent. of the mass of the solution. It is obvious that the mixture of medium and hydrochloric acid contains the least, that of medium and acetic acid the greatest, and that of medium and lactic acid an intermediate mass of undissociated acid. Pending an examination of the results obtained with other acids and media it would be premature to make a definite statement, but the results already obtained certainly suggest, that, when equivalent quantities are considered, highly dissociated acids are more effective in inhibiting the growth of *B. coli* than are those which are less dissociated. Further the final reaction reached by the culture depends rather upon the true acidity produced than upon the mass per cent. of acid present in the medium.

Examination of the forms of the curves expressing the reaction resultants of the various mixtures of media and acids reveals further signs of specificity.

While for hydrochloric and lactic acids the final reactions of the media become more acid, as the initial acid reaction of the medium increases, with acetic acid the reverse is obtained. For the former acids, it is evident that the amount of acid produced in the mixture is not rapidly diminishing in proportion to the rise of initial acid reaction, while with acetic acid a rise of initial acidity is accompanied by a marked diminution of acid formation.

It must be concluded that no attempts at diagnosis of the varieties of *B. coli* by means of their final reactions in a given medium can safely be made, since all strains and both varieties of the organism appear to react in the same manner when exposed to similar conditions. Also the final reaction attained is dependent upon the medium used, and the nature and amount of acid added to it.

In nearly every experiment it was found that at or near the expiration of a period of 168 hours from inoculation the P_H of the cultures showed a slight decrease of acidity. Possibly this is caused by the accumulation of metabolites produced during fermentation, and the phenomenon is graphically expressed in those figures in which the reaction resultant curve crosses, and passes to the left of, the initial reaction axis *AB*.

TABLE IX. *Glucose phthalate + hydrochloric acid, inoculated with* H_4 18 *hours old.*

Tube	*N*/5 acid per 10 cc. solution	Result	Time in hrs.	P_H Initial	4	8	24	48	72	168
1	0	+		7·11	6·89	5·60	5·60	5·6	5·65	5·70
2	0·3	+		6·80	6·73	5·43	5·41	5·27	5·11	5·11
3	0·4	+		6·69	6·64	5·36	5·20	5·16	5·07	5·09
4	0·5	+		6·52	6·50	5·29	5·21	5·13	5·05	5·06
5	0·7	+		6·27	6·27	5·75	5·34	5·27	5·04	5·01
6	0·8	+		6·13	6·13	5·30	5·01	5·13	4·97	4·97
7	0·9	+		5·95	5·95	5·04	5·03	5·01	4·94	4·97
8	1·0	+		5·77	5·77	5·38	4·94	5·04	4·89	4·94
9	1·5	+		5·01	5·01	5·01	4·90	5·85	4·75	4·75
10	1·8	+		4·96	4·96	4·90	4·82	4·68	4·68	4·68
11	2·4	–		4·41	4·41	4·41	4·41	4·41	4·42	4·42

6. A number of "retardation" experiments were performed, and it was conclusively proved that in addition to the total inhibition of growth produced by the addition of an acid to a medium, smaller quantities of acid caused a well-marked retardation of the fermentation of *B. coli*. For any given acid and medium an increase in the amount of acid added resulted in a longer period of retardation before the solution reached its final acid

reaction. With either glucose peptone, or glucose phthalate, to which no acid had been added, signs of fermentation were always visible within four hours of inoculation, but when acid insufficient to produce total inhibition of growth was added the period of retardation was sometimes prolonged to nearly 48 hours. Table IX shows the results obtained in a typical "retardation" experiment.

Summary.

1. All strains of *B. coli*, whether of human or of bovine origin behave similarly when exposed to similar conditions.

2. The degree of acidity of the final reaction produced by a culture of *B. coli* cannot be used for diagnostic purposes. This value is not a "physiological constant," but depends upon:

(*a*) The initial H-ion concentration of the medium in which fermentation occurs.

(*b*) The composition of the medium—especially the degree to which it is "buffered."

(*c*) The nature of the acid used to produce the initial reaction of the medium.

3. When the amount of acid added to a medium is insufficient completely to inhibit the fermentation of *B. coli* therein, a definite latency of growth results. For any given medium, a greater initial addition of acid causes a longer latent period.

4. Each acid appears to have a specific effect in inhibiting the growth of *B. coli* in a given medium, highly dissociated acids being more strongly inhibitory than those undergoing a less degree of dissociation.

5. For a mixture of any given medium and acid there appears to be a definite "critical point," beyond which the slightest rise in the degree of acidity results in a complete inhibition of the growth of *B. coli*. The critical points approximately determined as a result of the foregoing experiments are:

2 per cent. glucose peptone	+	hydrochloric acid			$P_H = 4{\cdot}27$
,, ,, ,,		lactic		,,	$P_H = 4{\cdot}47$
,, ,, ,,		acetic		,,	$P_H = 4{\cdot}68$
Glucose	+ phthalate +	hydrochloric acid			$P_H = 4{\cdot}55$
,, ,,		lactic		,,	$P_H = 4{\cdot}73$
,, ,,		acetic		,,	$P_H = 4{\cdot}83$

The specificity of action of the acids in these mixtures is also illustrated by the form of the reaction-resultant curve obtained in any given case.

In conclusion I wish gratefully to acknowledge my indebtedness to Captain G. G. L. Wolf, R.A.M.C., at whose suggestion this research was undertaken, for much valuable advice and guidance during the prosecution of the work.

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XXXI. CARNOSINE, CONSTITUTION AND SYNTHESIS.

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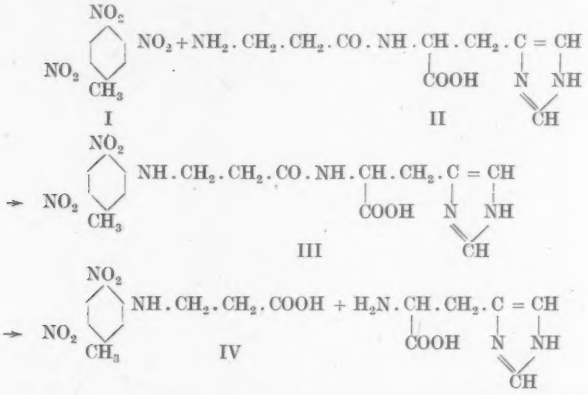
While engaged on work relating to the physiological behaviour of the trinitrotoluenes we observed that the β- and γ-isomerides condense with amino-acids on boiling in dilute alcoholic solution; the amino-acid becomes attached to the benzene ring by its amino-group, in replacement of a reactive nitro-group, which is eliminated. This reaction does not occur with imino-groups, whether present in a glyoxaline ring or in peptides, but does take place with the free amino-groups of peptides. The resulting compounds which are therefore *N*-dinitrotolylamino-acids resist boiling with concentrated hydrochloric acid and hence this reaction provides a suitable means of determining the constitution of peptides, for it is only necessary to combine the peptide with β- or γ-trinitrotoluene and hydrolyse the reaction product, when only those amino-acid radicles which originally had free amino-groups remain attached to the benzene ring, and these can therefore be identified. We applied this reaction to the dipeptide carnosine. Gulewitsch [1911] has shown that this base, one of the most important of those occurring in muscle extracts, is an anhydride of histidine and β-alanine, but whether β-alanyl-histidine or histidyl-β-alanine, remained undecided.

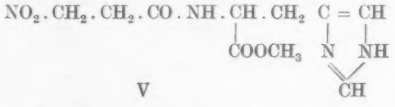
This question had formerly occupied one of us in conjunction with Dr A. J. Ewins, and some experiments on the hydrolysis of deaminocarnosine, which the latter carried out in 1914, and has kindly allowed us to quote below, indicated that carnosine is β-alanylhistidine. It was, however, impossible to isolate the deaminocarnosine in a pure condition or to obtain more positive evidence of the deamination by isolating β-hydroxypropionic or acrylic acid after hydrolysis. The condensation product of γ-trinitrotoluene with carnosine on hydrolysis furnished dinitrotolyl-β-alanine in a

pure condition, without any trace of the corresponding histidine compound, thus proving carnosine to be β-alanylhistidine and this result was communicated to the Biochemical Society at a meeting at the Lister Institute in December, 1917.

The reactions involved may be formulated as follows:



γ-Trinitrotoluene is 2 : 4 : 5-trinitrotoluene, in which the 5-nitro-group is labile, as shown by the production of 2 : 4-dinitrotoluene by the action of ammonia, diazotising and boiling with alcohol. Its condensation product (III) with carnosine (II) is a carboxylic acid and (owing to the presence of the glyoxaline ring) also a base; it is therefore insoluble in ether. It is hydrolysed by boiling acids to IV, a carboxylic acid without basic properties, and soluble in ether, which was found to be identical with the substance obtained by condensing β-alanine with γ-trinitrotoluene. This result encouraged us to attempt the synthesis of carnosine. The action of ammonia on β-chloropropionylhistidine (prepared like the α-bromo-isocaproyl derivative of Fischer and Cone [1908]) did not yield the desired result. We therefore condensed histidine methyl ester with β-nitropropionyl chloride, and reduced the resulting product (V). After hydrolysis of the



ester grouping this yielded carnosine, which we eventually isolated with considerable loss as the copper salt and identified. Our single experiment on a small scale yielded only 19 mg. of the copper salt, and being mainly occupied

with questions of a more practical kind, we did not repeat the experiment on a larger scale. Subsequently our attention was called to a paper by Baumann and Ingvaldsen [1918] who had also determined the constitution of carnosine and obtained the synthetic base in much larger quantity and in the free state. As our methods both of establishing the constitution, and of synthesis, differ from those given by Baumann and Ingvaldsen, we consider it advisable to publish our rather incomplete experiments.

A. Constitution.

Experiments by Dr A. J. Ewins (1914). 0·1 g. of carnosine was kept for two days at room temperature with one molecular proportion of silver nitrite and of hydrochloric acid. After filtration from silver chloride, a small amount of silver was removed from the filtrate by hydrogen sulphide. The solution was then concentrated to a small bulk and alcohol was added, when a gum separated. This was dissolved in concentrated hydrochloric acid and boiled for 6 hours, then evaporated to dryness. After redissolving in a little water and filtering from an amorphous impurity, the filtrate on standing deposited large colourless crystals of histidine dihydrochloride, m.p. 260°. The salt gave a picrate, m.p. 90–93°. In another experiment with 1 g. of carnosine and $1\frac{1}{2}$ molecular proportions of silver nitrite and hydrochloric acid numerous attempts were made to crystallise the deaminocarnosine, without success; nor did it yield a crystalline phosphotungstate. The yield of histidine dihydrochloride was from the main bulk 0·55 g. or 55 per cent. of the theory.

Action of γ-trinitrotoluene on carnosine. 0·5 g. of γ-trinitrotoluene and 1·0 g. of carnosine (two mols.) were dissolved in slightly diluted alcohol and boiled under a reflux condenser for one hour. As in all similar cases the liquid soon became deep yellow in colour. After evaporation on the water-bath, absolute alcohol was added and the evaporation was repeated to remove the small amount of water present. A bright yellow amorphous solid separated, very little soluble in absolute alcohol, even on boiling, but readily soluble in water. When damp with alcohol it was very deliquescent, but no longer so after washing with dry ether. This substance may be regarded as the carnosine salt of the condensation product of carnosine with γ-trinitrotoluene (III). It was dissolved in water and acidified with acetic acid when the condensation product itself was thrown down as a bright yellow precipitate, resembling the corresponding product from histidine and γ-trinitrotoluene. It was quite insoluble in ether, very little soluble in absolute alcohol and boiling water,

much more readily in boiling 50 per cent. alcohol. On concentrating its solution in the latter solvent it separated in yellow granules but could not be obtained distinctly crystalline.

Hydrolysis of the condensation product; 0·3 g. was boiled for 5 hours with 30 per cent. aqueous sulphuric acid under a reflux condenser, when all but a trace of dark coloured resinous material had dissolved. The liquid was extracted with ether which removed a yellow substance. The ethereal solution was extracted with a small quantity of dilute ammonium carbonate and the resulting deep yellow alkaline extract acidified with sulphuric acid when a yellow solid separated. The latter crystallised from hot water or dilute alcohol in tufts of small, bright yellow needles, melting at 166°. The substance was evidently not the condensation product of γ-trinitrotoluene with histidine, which is insoluble in ether and decomposes violently at 265°, but rather the condensation product of γ-trinitrotoluene with β-alanine. This was synthesised and isolated in a similar manner, and found to be identical with the substance from carnosine. A mixture of the two melted at 166°. The synthetic compound was analysed.

0·1291 g.; 0·2102 CO_2; 0·0500 H_2O. C = 44·4, H = 4·3.
Calc. for $C_{10}H_{11}O_6N_3$. C = 44·6, H = 4·1.

B. Synthesis.

We first attempted the synthesis in the way used by Fischer and Cone [1908] for the synthesis of leucylhistidine, and accordingly acted on histidine methyl ether with β-chloropropionylchloride. The crude condensation product, which could not be crystallised, was treated with ammonia at various temperatures; this did not result in the formation of any appreciable quantity of carnosine, but apparently of an unsaturated compound. With copper carbonate we obtained indications of carnosine copper salt, but the use of β-chloropropionyl chloride had to be abandoned. Baumann and Ingvaldsen found the same difficulty but were successful when they used the chloride of β-iodopropionic acid. We met this difficulty by using the chloride of β-nitropropionic acid, and reducing the compound formed. From glycerol we prepared successively glyceric acid, β-iodopropionic acid and β-nitropropionic acid, the last named according to Lewkowitsch [1879]. β-Nitropropionyl chloride had not been prepared hitherto; we heated the acid with excess of thionyl chloride on the water-bath for two hours, removed the excess of thionyl chloride by gently warming in a vacuum, and then distilled the residue in small quantities at a time by plunging the flask evacuated to

10 mm. into a metal bath previously heated to 160°. The object of this precaution was to limit the duration of heating, as otherwise the chloride is apt to decompose. We thus obtained an almost colourless liquid, B.P. 123°/10 mm.

0·1425 g.; 0·1511 AgCl. Cl = 26·2
Calc. for $C_3H_4O_3NCl$. Cl = 25·8.

The β-nitropropionyl chloride was next condensed with histidine methyl ester. 2 g. of the acid chloride in 20 cc. of dry chloroform was added gradually, with vigorous shaking, to a chloroform solution of rather less than two equivalents of the ester, cooled in a freezing mixture. As soon as the mixture ceased to give an alkaline reaction, the addition was discontinued and the chloroform solution of the condensation product was decanted from the histidine methyl ester monohydrochloride, which had separated. It was found impossible to isolate the condensation product itself since it decomposed on gently warming the solution, and quite appreciably during a few hours at ordinary temperatures. It was therefore at once reduced, by adding excess of stannous chloride in dilute hydrochloric acid to the above mentioned chloroform solution; the chloroform was removed by very gentle warming under reduced pressure. The temperature was then gradually raised and finally maintained at 100° for one hour. After cooling, the tin was removed as sulphide, and the filtrate was made slightly alkaline and kept over night to hydrolyse the ester. On evaporation to dryness the residue, consisting largely of sodium chloride, was extracted with methyl alcohol, which was distilled off, leaving a viscid residue of crude carnosine. As it could not be crystallised, we precipitated with phosphotungstic acid and obtained on regeneration 0·5 g. of a base, which on digestion with copper carbonate gave a deep blue solution. The latter was concentrated and deposited first an amorphous copper salt which was removed from time to time. Finally, on keeping the concentrated mother liquor for some days, ill-formed six-sided crystals separated, which were recrystallised and then yielded deep blue hexagonal plates, characteristic of the copper salt of carnosine. We finally only obtained 19 mg. of the pure salt. The recrystallisation is very troublesome and attended with great loss, as was pointed out by Gulewitsch and Amiradzibi [1900]. Perhaps we would have done better to attempt the crystallisation of the nitrate of the base regenerated from the phosphotungstate. The copper salt was dried *in vacuo* over sulphuric acid for some months, and was then analysed by Pregl's micro-Kjeldahl method [see Abderhalden, 1912].

I. 2·85 mg.; 2·65 cc. $N/70$ HCl. N = 18·6,

II. 5·7 mg.; 5·0 cc. $N/70$ HCl. N = 17·5.

For the sake of comparison natural carnosine copper salt was also analysed.

3·25 mg.; 3·0 cc. $N/70$ HCl. N = 18·5.

Calc. for $C_9H_{14}O_3N_4 . CuO$. N = 18·3.

We also determined the rotation. It was considered inadvisable to attempt the removal of the copper from so small a quantity. Hence we merely dissolved the salt in excess of dilute acid.

9 mg. Cu salt in 200 mg. 13 per cent. sulphuric acid gave a pale blue solution containing 6·6 mg. carnosine.

$c = 3·3$ per cent.; $l = 0·5$ d.m.; $\alpha_D = + 0·15°$; $[\alpha]_D = + 9°$.

Gulewitsch [1913] gives for carnosine nitrate dissolved in excess of nitric acid, $[\alpha]_D = + 12°$ to 13°.

We desire to express our thanks to Dr J. C. Drummond, of the Cancer Hospital, for the use of a micro-polarisation apparatus and kind help in the above determination.

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XXXII. ACCESSORY FACTORS IN THE NUTRITION OF THE RAT.

By ARTHUR HARDEN and SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received October 30th, 1918.*)

The researches of McCollum and Davis have revealed the fact that rats cannot grow or exist on an artificial diet unless two principles as yet chemically unknown are present. These were designated by McCollum and Kennedy [1916] as fat-soluble A and water-soluble B. The former is associated in nature mostly with fats of animal origin; the latter is soluble in water. McCollum and Kennedy have further found that the water-soluble B if not identical with the antineuritic factor is at least closely associated with it, and this has been confirmed by Drummond [1917]. More recently McCollum and Pitz [1917] and Pitz [1918] have expressed the view, based on observations made on guinea-pigs and rats, that scurvy is not a disease produced by the deficiency of an accessory factor and that the scorbutic condition produced in guinea-pigs by dietetic means is due to other unfavourable points in the diet. In a previous communication [1918, 2] we have already discussed their experiments on the guinea-pigs and have shown that their conclusions were not warranted by the experimental evidence brought forward in their papers. We hold the view that scurvy is a disease brought about by the dietetic deficiency of a principle distinctly different in its physical and other properties from the antineuritic and the fat-soluble A factors. It is evident from the extensive researches of Osborne and Mendel, and McCollum and his collaborators that by omitting the addition of antiscorbutics to their purified diet, no scorbutic lesions can be produced in rats, such as are found in human subjects, monkeys and guinea-pigs, when they subsist on diets deficient in antiscorbutics. Guinea-pigs when put on oats, bran and autoclaved milk show scorbutic symptoms 14–21 days after the commencement of the experiment. Our own observations on monkeys [1919] show that these animals manifest scorbutic lesions after $2\frac{1}{2}$–3 months' subsistence on a diet free from

antiscorbutics. As regards human subjects there appear to be a few apparently reliable clinical records of cases in which, owing to adverse circumstances, the subjects have been entirely deprived of antiscorbutics for a considerable time. Judging from these, 6–8 months seems to be the time necessary for the subject to exist on a deficient diet before the disease is well declared. In perusing the literature one finds, however, that rats have been kept by various experimenters for a considerable length of time on scorbutic diets without showing any signs of scurvy. The experiments we are about to describe show nevertheless that rats existing on a scorbutic diet, although capable of gaining in weight and reproducing themselves, without any apparent manifestation of pathological symptoms for months, do not thrive so well as animals which have their diets supplemented with an antiscorbutic. This suggests that although rats are not very susceptible to scurvy they cannot absolutely dispense with antiscorbutics without restriction of their normal development.

Experimental.

Our investigations were carried out on several groups of rats receiving a purified basal diet of starch, caseinogen and inorganic constituents and the various accessory factors. The basal diet was made up as follows:

Starch	75 per cent.
Caseinogen	20 ,,
Salts	5 ,,

The salt mixture was the same as that employed by McCollum and his collaborators in their researches and had the following composition:

NaCl	1·73 g.	$CaH_4(PO_4)_2\,2H_2O$	5·40
$MgSO_4$ (anhydr.) ...	2·66	Calcium lactate ...	13·0
$NaH_2(PO_4)H_2O$ ...	3·47	Ferric citrate ...	1·18
K_2HPO_4	9·54		

The fat-soluble A factor was supplied in the form of butter-fat purified by centrifuging the melted fat and decanting the supernatant layer. The antineuritic factor was introduced by adding autolysed yeast. The antiscorbutic was added in the form of lemon juice from which the citric and other organic acids had been removed by the method we have already described in a previous communication [1918, 1].

For the sake of convenience we shall designate in this paper the diets of the various groups by the accessory factors they contain, using the letters

A, B, and C for the fat-soluble A, antineuritic, and antiscorbutic factors respectively. The rats were kept separately in cages; couples belonging to the same group were brought together only occasionally for mating purposes. The food was supplied to them fresh in balls every day and the remains of the previous day were weighed daily. The drinking water was changed twice daily.

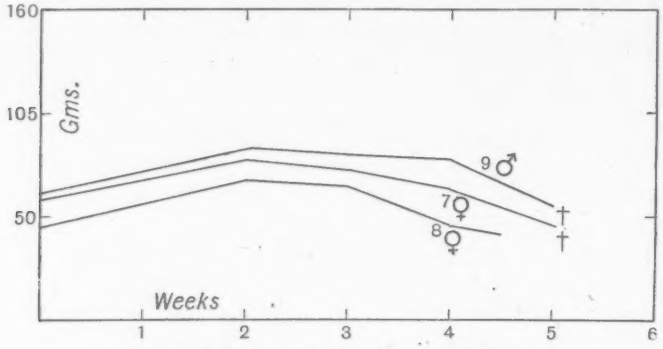

Fig. 1. Weight curves of 3 rats on an AC diet.

Fig. 1 represents the weight curve of 3 rats kept on an AC diet. The daily ration of each of these rats was made up as follows:

Basal mixture	10 g.
Antiscorbutic equiv. to lemon juice ...	5 cc.
Centrifuged butter fat	1·8 g.
Olive oil	1·6 g.

The above ingredients were incorporated in a single ball, the olive oil being added in order to obtain a convenient consistency in the paste. From $\frac{1}{2}$–$\frac{3}{4}$ of the daily ration was consumed by the animals.

It is seen from the weight curves that none of the animals has gained appreciably in weight during any period of the experiment. This becomes more marked as the rats were at an age when development under normal conditions progresses rapidly. After about three weeks the animals commenced losing in weight and died five weeks after the commencement of the experiment. Rat No. 8 was killed by the buck after $4\frac{1}{2}$ weeks when left with it in a cage for mating purposes. Rats Nos. 7 and 9 showed decided neuritic lesions before death. Rat No. 7 first showed marked paralysis in the hind limbs four days before death. This condition became gradually worse. Rat No. 9 showed even more striking symptoms. The following was its condition three

days before death[1]. Rotated as ingoing corkscrew. Position of left ear approximated left shoulder. Left eye to the ground. Tone increased on left side. Body was bent anticlockwise, *i.e.* concavity to the left. No circus movements observed. Animal was able to use fore and hind legs for grasping objects apparently without incoordination. Could not walk in a normal manner. It stood on a wide base. Difficulty appeared to be due to the increase in tone of muscles of limb. A day later the animal showed circus movements and died on the subsequent day. Portions of the sciatic nerves of rats No. 7 and No. 9, teased and treated with osmic acid, were examined histologically by Miss F. M. Tozer. In Miss Tozer's opinion the greater part of the nerve of rat No. 9 was sound. There were however a fair number of curiously eroded abnormal looking fibres concerning which in the absence of further evidence it is difficult to give a definite opinion. Their appearance is possibly suggestive of a very early stage of degeneration. The teased preparation of the nerve of rat No. 7 showed no degenerating fibres.

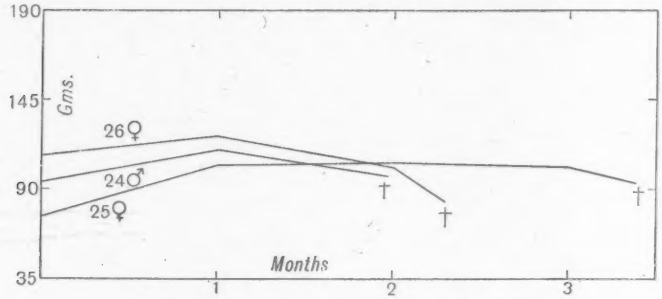

Fig. 2. Weight curves of 3 rats on a purified B diet.

Fig. 2 represents the weight curves of three rats fed on a B diet. The daily ration of these animals was as follows:

Basal mixture	12 g.	Water	2½ cc.
Autolysed yeast ...	5 cc.	Olive oil	2½ cc.

From ½–¾ of this ration was consumed by the animals daily. All these animals succumbed to the fat-soluble A factor deficiency within a period varying from 2 to 3½ months. During the first month they all gained in weight. Their weight then became more or less stationary, but the animals declined a little before death. Two of these rats, namely 24 and 26, showed xerophthalmia in acute form.

[1] We are indebted to the late Dr F. E. Batten for this description of the condition of the animals.

That the antiscorbutic factor does not fulfil the same physiological function as the fat-soluble A factor is seen from Fig. 3 which represents the weight curves of three rats fed on a BC diet. The daily ration of this group was made up as follows:

Basal mixture	12 g.
Antiscorbutic equiv. to lemon juice	5 cc.
Autolysed yeast juice	5 cc.
Olive oil	0·75 cc.

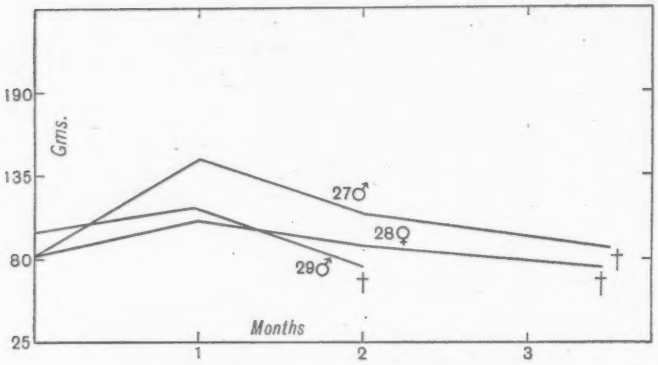

Fig. 3. Weight curves of 3 rats on a pure BC diet.

About three-quarters of the daily ration was consumed by the rats. The weight curves of this set do not differ much from those of the previous group. Rat 29 died two months and rats 27 and 28, 3½ months after the commencement of the experiment. They all developed xerophthalmia. It is evident from this experiment that the addition of an antiscorbutic to a diet lacking the fat-soluble A does not rectify the deficiency.

In the last two experiments the caseinogen employed was previously extracted with ether and alcohol. Experiments of the same character as these two were made in which the caseinogen had not been previously purified. The results obtained are of sufficient interest to be recorded. Fig. 4 shows the weight curves of a group of rats fed on B diet with unpurified caseinogen and Fig. 5 represents the same of a group of rats fed on a BC diet with unpurified caseinogen.

The general characters of these curves are the same as those of the animals fed on similar diets with purified caseinogen. The main difference lies

in the prolonged period of time during which the animals lived on the experimental diet. After about two months, growth was entirely suspended and a decline in weight was observed towards the end of the experiments. Many of these animals displayed xerophthalmia. During the progress of the experiment the buck of each set was on several occasions mated with the does, but no litters were produced. This prolonged sustenance on a diet greatly deficient in the fat-soluble A factor afforded us the opportunity of observing certain marked histological changes in the *post-mortem* tissues of these animals which are of decided interest. We shall describe and discuss these results in another communication.

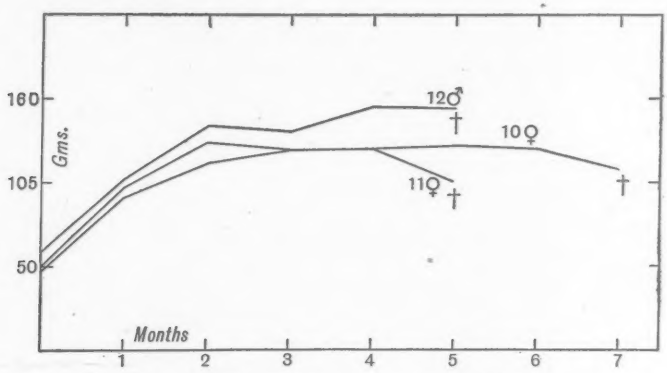

Fig. 4. Weight curves of 3 rats on a crude B diet.

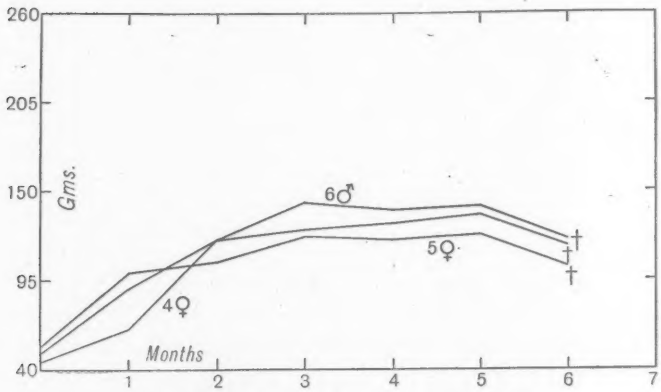

Fig. 5. Weight curves of 3 rats on a crude BC diet.

Two groups of rats were also fed on AB and ABC diets. These were made up as follows:

	AB	ABC
Basal mixture	10 g.	10·6 g.
Purified butter	1·85 g.	1·7 g.
Autolysed yeast	5 cc.	5 cc.
Olive oil	2·5 cc.	0·25 cc.
Antiscorbutic equiv. to lemon juice	—	5 cc.

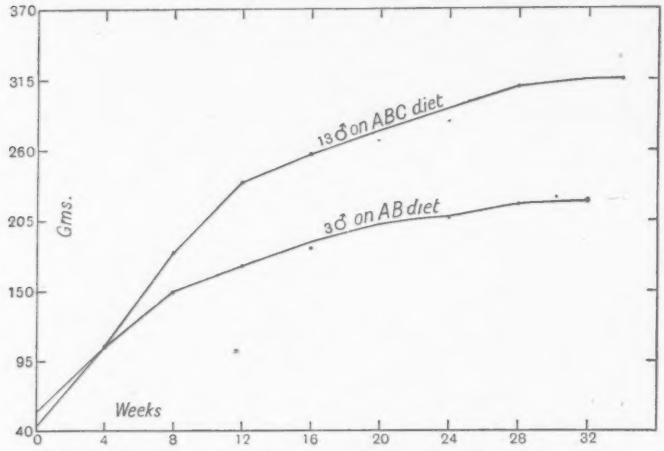

Fig. 6. Weight curves of 2 ♂ rats on ABC and AB diets respectively.

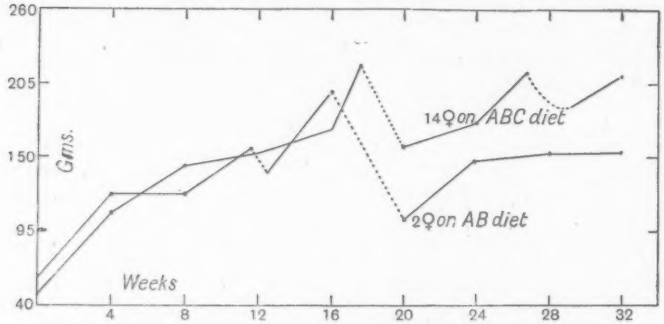

Fig. 7. Weight curves of 2 ♀ rats on ABC and AB diets respectively.
---- indicates that no weighings were made owing to the birth of a litter.

These two groups grew satisfactorily and reproduced themselves. The ABC group however flourished decidedly better than the AB group. This can be seen from Figs. 6 and 7 which represent the weight curves of animals

belonging to those two groups. Fig. 6 represents the weights of the two respective bucks and Fig. 7 of the two does on the two diets. In both cases the difference in growth is decidedly marked. This observation does not appear to be accidental as similar observations have been made in this laboratory on other animals kept on similar diets and so far no deviation from the above behaviour has been recorded.

If animals kept on the AB diet do not develop to their fullest capacity without having the antiscorbutic added in their diet, it would appear that rats are susceptible to an antiscorbutic deficiency although they do not develop definite lesions. Further investigations will definitely decide this point.

Summary.

1. An antiscorbutic does not fulfil the physiological function of the fat-soluble A when it replaces it in the diet of the rat.

2. The authors confirm the observation of McCollum and his colleagues and of Drummond that by depriving rats of the antineuritic factor a dietetic deficiency is brought about as a result of which neuritic lesions are produced in the animals and a fatal termination ensues if the diet is not rectified in time.

3. Rats subsisting on a diet containing the antiscorbutic as well as the water-soluble and fat-soluble A factors grow better than rats from the diet of which the antiscorbutic factor is absent.

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XXXIII. THE ANTISCORBUTIC VALUE OF CABBAGE. I. THE ANTISCORBUTIC AND GROWTH PROMOTING PROPERTIES OF RAW AND HEATED CABBAGE.

By ELLEN MARION DELF,

WITH AN APPENDIX

By FRANCES MARY TOZER.

ON THE HISTOLOGICAL DIAGNOSIS OF EXPERIMENTAL SCURVY.

From the Lister Institute of Preventive Medicine.

(*Received November 12th, 1918*)

The antiscorbutic properties of fresh vegetables are well known and cabbages of all kinds are especially valuable in this respect. At the present time, owing to the scarcity of fresh fruit, it is important that the best use should be made of any available green vegetables. Most vegetables require cooking in order to make them digestible and palatable enough for human consumption. It is known, however, that the antiscorbutic value of both vegetables and fruit is diminished by cooking, and the present investigation is an attempt to estimate the loss in the antiscorbutic properties of fresh cabbage after exposure to heat at various temperatures and for different intervals of time.

In order to have some basis of comparison it has been necessary to make preliminary experiments using varying amounts of fresh raw cabbage as the sole antiscorbutic in an otherwise adequate diet. Guinea-pigs were chosen as experimental animals and the standard diet selected was a mixture of rolled oats and bran *ad libitum*, and water. With small rations of cabbage (5 g. daily and under) 60 cc. of milk previously heated in an autoclave for one hour at 120° C. was also given daily. This was found to improve the nutritive value of the diet and encourage growth without influencing the onset of scurvy.

The Antiscorbutic Value of Raw Fresh Cabbage.

Experiments of Holst and Frölich [1912] indicate that normal growth of young guinea-pigs can be secured on a diet of fresh cabbage leaves and some kind of cereal food (oats, rice, rye bread or white bread). The change in body-weight relative to the initial weight varied considerably with the amount of cabbage given (see Table I). The only ration which gave both a fairly satisfactory growth and complete protection from scurvy was one of 30 g. daily, but probably younger animals would have made growth on a smaller ration. However, the animals were evidently not under very favourable circumstances, for out of 56 cases cited [Holst and Frölich, 1912, Table IV A], 27 animals died from illness—sometimes of an unknown character, but often from pneumonia or enteritis. Nearly complete protection from scurvy was given by a daily ration of only 1 g., but the animals survived for only a short time with much loss of weight, and in the further work of these authors on dried and heated vegetables, 30 g. of fresh cabbage daily was chosen as the standard ration.

Table I. *Summary of Results of experiments of Holst and Frölich with fresh cabbage (calculated from their paper* [1912, Table IV A, p. 64, and pp. 106–112]).

Ration given	Mean initial weight of animals in g.	No. of animals	Time of experiment in days	Total gain or loss in weight %	Result
30 g. white cabbage and oats and bran *ad lib.*	328	5	100–200	+33	No scurvy
15 g. white cabbage and white bread	507	3	33–105	−25	do.
10 g. white cabbage and white bread	475	4	46–113	−22	Traces of scurvy in one case
10 g. white cabbage and rice (husked)	676	4	28–72	−41	do. do.
25 g. white cabbage and oats and bran *ad lib.*	342	2	15–28	−50	No symptoms in one case, loose teeth in one case
1 g. white cabbage and oats and bran *ad lib.*	307	3	47–56	−50	No symptoms in one case, loose teeth in one case

In our experiments, care has been taken to give the animals the best conditions possible. The laboratory in which they were kept was well ventilated and warmed during the winter (60°–65° F.). Most of the animals were bred on the premises and were well fed and housed from the first. Shortly before starting on an experiment, the animals were placed in separate cages on a litter of peat and were kept under observation for a few days. If the

general condition and the weight curve were satisfactory, the experiment was started when a suitable weight had been attained (about 330–350 g.) but doubtful animals were rejected for the purposes of these experiments.

In the case of animals under experiment, all the rations were given by myself or by fully trained assistants and a daily record was kept of the amount actually consumed. It was generally possible to ensure that the whole of the cabbage ration was eaten, hand feeding being introduced where necessary. All the cabbage was given as green leaf, avoiding both the thickest part of the midrib, and the whitish inner leaves of the heart. In this respect the cabbage used differs from that given by Holst and Frölich who used "white" cabbage (*i.e.* the winter cabbages with large, compact, white hearts) and did not apparently select definitely either the inside or outer green leaves. In our experiments with small rations, especial care was exercised in selecting only the healthy green parts of the turgid leaf to ensure uniformity as far as possible.

Clean water or milk, as the case might be, was given twice daily, and almost every day the animals were allowed to run about on bench or table for exercise. We believe that this regular exercise, even when only for brief periods of time, has a very beneficial effect on their general health and well being. It also allows observation of any inactivity or lameness which is a frequent precursor of the onset of scurvy. For the most part the animals were remarkably healthy; out of the 27 animals on fresh cabbage quoted in Table II only one died from an unknown cause before the completion of the three months of the experiment. In spite of every precaution, however, and the daily disinfection of all the vessels used for feeding, some unexplained deaths occurred occasionally, especially on the smaller limiting ration of cooked cabbage. This may be caused by lowered resistance to chance infection resulting from an insufficient margin of antiscorbutic in the diet to ensure perfect robustness.

Animals of about 330 g. initial weight have been selected as far as possible and the usual duration of an experiment was 90 days. The cereal food given throughout was a mixture of rolled oats and bran which was readily and often greedily eaten. The animals were weighed three times weekly and carefully examined every 5 or 6 days, or oftener when the onset of scurvy was expected.

The symptoms to be found in scorbutic animals have been described in a communication by Chick, Hume and Skelton [1918], but may be recapitulated here. They are: (1) Soreness of joints. (2) Lameness,

with or without general inactivity. (3) The adoption of various typical "scurvy positions" when in repose. In these positions, the limb affected is often held up and in the "face-ache" scurvy position the head is lowered and the neck stretched out (see Plate I, figs. 2 and 3). Post-mortem examination of animals which have frequently adopted the "face-ache" position shows that the teeth are loose and the jaws so fragile as to break with the slightest pressure. (4) Severe cases of long standing have swollen knees often fractured at the epiphysial junction of the tibia, the back limbs being then almost helpless. Photographs of some typical scurvy positions are shown in Plate I.

The post-mortem signs of scurvy are well known and comprise chiefly (1) haemorrhages in the subcutaneous tissue, muscles, periosteum or viscera, (2) fragility of bones, with fracture in severe cases, (3) ridges or swellings on the rib-junctions, (4) loosened teeth set in a fragile jaw and (5) wasted tissues. A systematic examination has been made by Miss F. M. Tozer of the minute structure of the rib-junctions of these and many other experimental animals. On the whole, her independent histological diagnosis falls into line with the symptoms observed during life and with the results of post-mortem examination. In general, it has been possible to distinguish histologically three principal stages in the course of the disease, which may be termed respectively incipient, definite and acute[1]. The incipient stage involves only slight histological abnormality (Plate II, fig. 2) corresponding to absence of definite symptoms in life and very slight evidence of scurvy or none at all at the post-mortem examination. Definite and acute scurvy implies marked disorganisation of the normal structure of the bone cartilage junction (in which growth appears to become arrested in chronic cases) and agrees usually with marked evidence of scurvy during life and at the post-mortem examination. Further details of these stages are given by Miss Tozer in Appendix I together with diagrammatic illustrations (Plate II, figs. 1–6).

Table II gives a summary of the results of the experiments with raw cabbage rations, fuller particulars being given in Table VII. With a 30 g. ration (Exp. 1) the amount of growth was much greater than that found by Holst and Frölich, although the average initial weights of the animals were very similar. The mean growth curve A (Fig. 1) obtained by averaging the body-weights from time to time of all the animals in Exp. 1 has been adopted as the standard of normal growth throughout this investigation. It is nearly identical with the mean curve of normal growth deduced by Chick, Hume and Skelton from the growth of twelve animals upon full

[1] See note, p. 446.

TABLE II. *Experiments with limiting rations of fresh cabbage* (see also Table VII).

Exp.	Ration of fresh cabbage g.	Number of animals	Mean initial weight g.	Total gain %	Time of exp. days	Symptoms in life	Post-mortem examination	Histology of rib-junctions	General conclusions
1	30	3	340	91	87–90	None	Not examined	Not examined	Complete protection
2	15	4	345	74	90	do.	No indications of scurvy	Normal	do. do.
3	5	3	323	26	90	Slight soreness	do. do.	Incipient scurvy	Protection
4	5	3	325	69	90	do.	do. do.	do.	do.
5	2·5	5	323	49	90	Soreness in 2 cases	Brittle leg bones in one case, brittle jaw in another	do.	do.
6	1·5	6	346	49	70–90	Soreness in 5 cases out of 6	Brittle bones in 2 cases	do.	do.
6 A	1·5 (citric acid)	4	330	70	90	Slight soreness after 49 days	2 normal, 2 slight symptoms	1 normal, 3 nearly normal	do.
7	0·5	4	331	18	67–90	Soreness, swollen joints	Three with fragile bones. All with rib-junctions ridged	1 normal 1 slight scurvy 1 chronic scurvy 1 severe scurvy	Scurvy in 3 out of 4 cases

In Expts. 1, 2, and 3 the rest of the ration consisted of water and rolled oats and bran *ad libitum*, the amount consumed daily being about 30 to 60 g. (see Table VII).

In Expts. 4–7 60 cc. milk autoclaved at 120° C. for 1 hour was substituted for the water.

normal diet. The average increase in body-weight is strikingly different in Expts. 1, 2, and 3, diminishing with the reduction of the cabbage ration, although the appetite was not impaired, and the grain consumed was not less (cp. Table VII and curves A, B and C, Fig. 1). In each case there was no symptom of scurvy during life, but the slight tenderness observed in the joints of animals fed on the 5 g. ration and the results of histological examination of their rib-junctions suggest a condition of incipient scurvy.

Since in these animals, growth was apparently limited by the extent of the cabbage ration apart from the incidence of scurvy, another series of animals were given a daily allowance of 60 cc. milk previously autoclaved for an hour at 120°, in order to improve the nutritive value of the diet. Previous experience of workers in this Institute has proved that the addition of this amount of autoclaved milk does not affect the onset of scurvy. Expts. 3 and 4 with a ration of 5 g. cabbage with and without milk, respectively, fully confirm this result. In both sets of animals a departure from the normal could be detected at histological examination of the rib-junctions, though in both sets the animals gave no marked signs of scurvy during life or at the post-mortem examination. The animals on the milk ration however grew much better and were heavier and in better condition throughout the experiment, which lasted three months.

No indications of scurvy were detected during life in most of the animals on 2·5 and 1·5 g. rations of cabbage to which autoclaved milk had been added (Expts. 5 and 6). The mean growth of these animals was less than that of those on the larger rations and the irregular growth on the smaller of these rations is probably significant (C and D, Fig. 2). On histological examination of the rib-junctions incipient scurvy was diagnosed in several cases. On the smallest ration of cabbage given, 0·5 g. (Exp. 7) no growth was made after the first 20 days (E, Fig. 2); three out of the four animals exhibited definite symptoms of scurvy and one died of acute scurvy after 67 days. There is little doubt that most young guinea-pigs would find this ration insufficient for maintenance of normal health. The limiting daily ration of fresh cabbage just protecting a young guinea-pig from definite symptoms of scurvy during life and maintaining health, upon the standard diet of oats and bran and autoclaved milk must therefore lie between 1·5 and 0·5 g. In the experiments of Holst and Frölich a ration of 1 g. fresh cabbage daily was found to give nearly complete protection from scurvy (Table I), and in the present communication this ration has been regarded as the probable minimum protective ration.

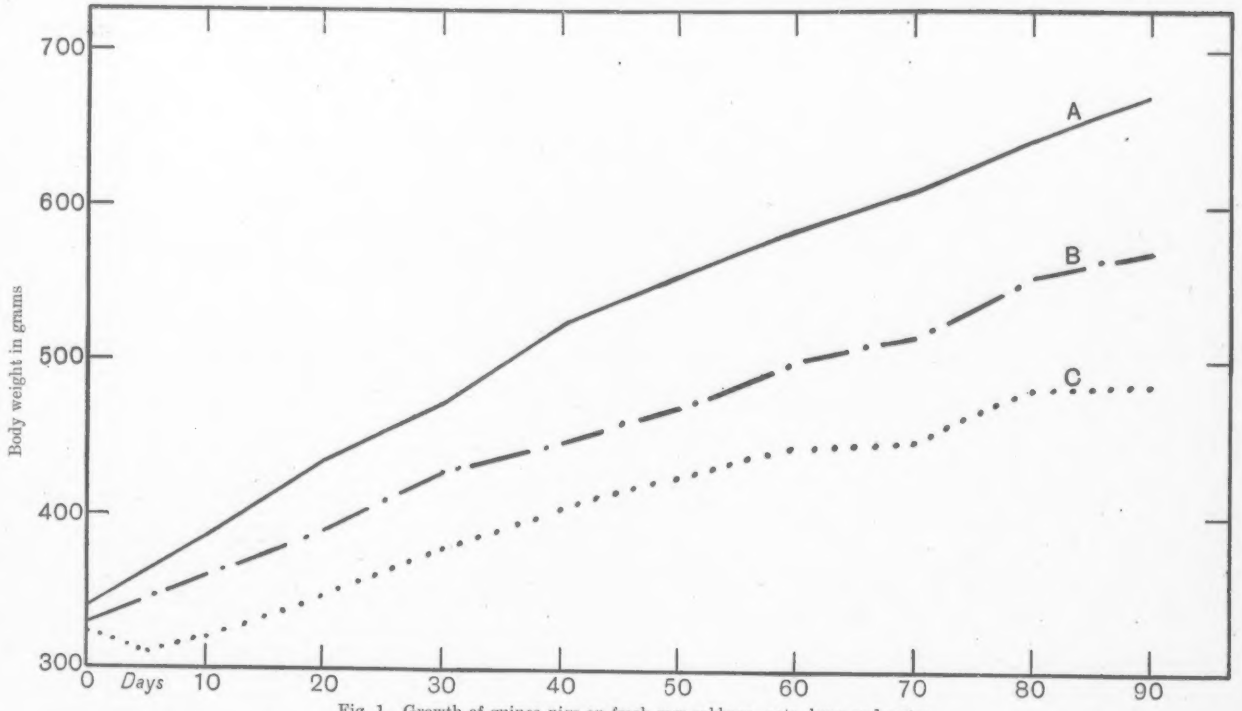

Fig. 1. Growth of guinea-pigs on fresh raw cabbage, oats, bran and water.

Curve A. Represents the standard growth curve of 3 animals upon a 30 g. fresh cabbage ration, oats, bran and water (Expt. 1, Tables II and VII).
,, B. Represents the average growth of 4 animals upon 15 g. fresh cabbage ration, oats, bran and water (Expt. 2, Tables II and VII).
,, C. Represents the average growth of 3 animals upon a 5 g. fresh cabbage ration, oats, bran and water (Expt. 3, Tables II and VII).

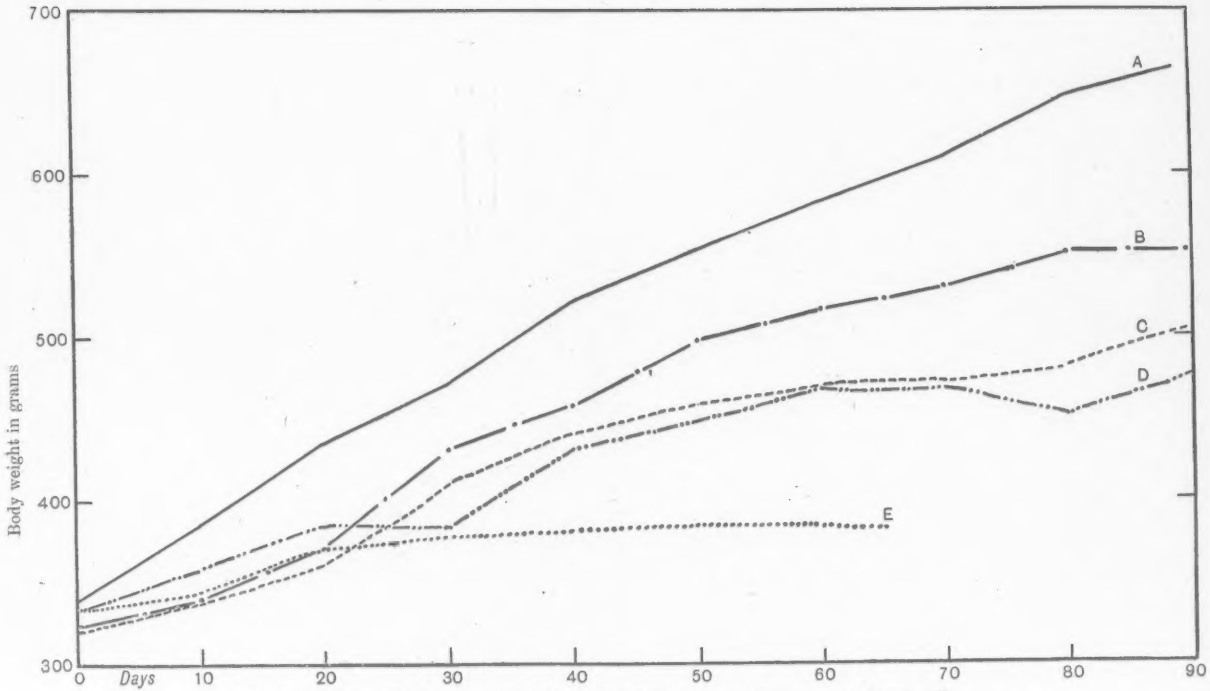

Fig. 2. Growth of guinea-pigs on fresh raw cabbage, oats, bran and autoclaved milk.

Mean weight curves of five groups of young guinea-pigs upon a diet of oats, bran, fresh cabbage and autoclaved milk daily. Each curve is obtained from the mean weights of all the animals in the experiment after definite time intervals (Tables II, VII, Expts. 1 and 4–7).

——— Curve A. Represents the standard growth curve of 3 animals upon a 30 g. cabbage ration (Expt. 1, Tables II and VII).

—•— ,, B. Represents the average growth curve of 4 animals upon a 5 g. cabbage ration with 60 cc. autoclaved milk.

– – – – ,, C. ,, ,, ,, 4 ,, 2·5 g. ,, ,, ,,

—••—•• ,, D. ,, ,, ,, 4 ,, 1·5 g. ,, ,, ,,

•••••• ,, E. ,, ,, ,, 4 ,, 0·5 g. ,, ,, ,,

From this series of experiments we may therefore conclude:

(1) That normal growth on a ration of 30 g. fresh cabbage under the conditions of our experiments involves an increase of body-weight of about 100 per cent. in 90 days.

(2) That smaller rations involve a corresponding diminution in the growth rate without however causing any definite symptoms of scurvy to appear during life unless the ration is reduced to 0·5 g.

(3) That apparently satisfactory growth and health are obtained with rations from 1·5–5 g., if 60 cc. autoclaved milk daily is added to the diet, but suggestions of soreness during life, together with departure from the normal in the histology of the bone cartilage junctions point to a condition of incipient scurvy.

(4) That on a 0·5 g. ration well-marked symptoms of scurvy may be expected.

(5) That a 1 g. ration of fresh cabbage may be regarded as the minimum for protection from the symptoms usually diagnosed as scurvy.

The Antiscorbutic Value of Cooked Cabbage.

The only information available as to the effect of heat on the antiscorbutic properties of cabbage is contained in the paper of Holst and Frölich [1912] to which reference has already been made. Their general conclusion was that heat has a distinctly destructive effect and that this destruction is greater at 110° and 120° than at 100° for the same time of heating, viz. 30 minutes.

They used steam under pressure in an autoclave for heating the material at 110° and 120°, but at 100° the leaves were either boiled in slightly salted water or they were placed in an Erlenmeyer flask and heated for the required time, the leaves being slightly salted. The animals are said to have licked up the juice which exuded during heating, and also to have eaten their ration of cabbage: they appear to have refused to eat it when unsalted.

Table III gives a review of their results, the averages of the initial and final weights of the period of survival being calculated from their figures (H. and F., Table IV B, pp. 66–70). After being heated for ½–1 hr. at 100° a ration of 30 g. appears to have protected the animals almost completely from scurvy, but there was little or (in one set) no gain in weight as would have been expected on a corresponding ration of fresh cabbage. With 30 g. cabbage heated ½ hr. at 110° one animal out of three had clearly scorbutic symptoms (loose teeth, haemorrhages and disorganised rib-junctions) whilst all animals were similarly affected when the same ration was heated for 1 hr. at 120°.

TABLE III. *Summary of Experiments of Holst and Frölich with Cooked Cabbage.* [See H. and F., 1912, Table IV B, pp. 66–70.]

Diet	No. of animals	Mean time of life	Mean weight in g. Initial	Final	Result
30 g. cabbage cooked 1 hr. in ½ % common salt at 100° C. and oats	5	87 days	351	352	Complete protection except in one case
30 g. cabbage steamed ½ hr. at 100° and white bread	3	153	390	488	Complete protection
	3	105	353	293	Protection with loss in weight in two cases. Scurvy in one case
30 g. cabbage steamed ½ hr. at 110° and white bread	3	96	393	327	Scurvy, with partial protection
30 g. cabbage steamed ½ hr. at 120°	3	45	387	268	All with severe scurvy

In contradistinction to their results, our experiments with a 15 g. ration of cabbage autoclaved at 110°–130° for one hour gave almost complete protection from scurvy in each case (see Table V). This difference in result is difficult to account for, unless it be due to the different technique, to the use of green rather than white cabbage or to heating without the addition of salt. The present experiments are in accordance with one another although they differ in results from those of the Norwegian investigators.

In order to get some basis for comparison with the experiments in which small rations of fresh cabbage were employed, we selected 5 g. as the standard ration for investigating temperatures below 100°, giving the animals (as in experiments with raw cabbage) an allowance of 60 cc. autoclaved milk daily. In many cases the animals voluntarily drank their milk, which was given morning and afternoon, but in cases of failing appetite through the onset of scurvy, or other disinclination, hand feeding with a glass syringe was adopted (cp. fig. 6, Plate I). As with the fresh cabbage experiments, only the outer green leaves were used for heating. We found it unnecessary to use salt in cooking. Most animals accepted the cooked cabbage readily and the rest were hand fed.

At 60°, 70° and 80° entire leaves were plunged into water contained in a French cooker surrounded by water at constant temperature. The temperature was kept under control and was observed at intervals during the time of cooking, and pieces weighing 5 g. were cut off after the cooking.

For the experiments at 90° and 100° heating was done in steam. Small pieces were cut from a fresh leaf, each weighing 5 g.; these were suspended singly from hooks on the inside of a saucepan lid which just fitted the steam chamber over a saucepan of boiling water. By adjusting the rate of boiling

and also the steam outlet, the temperature of this chamber could be kept at 90° or 100° as required. In order to determine whether immersion in water at 100° itself caused any additional loss of antiscorbutic properties, a further set of animals were given a 5 g. ration taken from whole leaves which had been plunged into boiling water and boiled briskly for 20 minutes. They were then lightly drained, cut up and an amount equal to the 5 g. ration weighed out from them, an additional 0·5 g. being allowed to compensate for the extra water taken up in this method of cooking.

In all cases the cooking was done either daily or on alternate days, the cooked pieces being kept moist and placed in a refrigerator until wanted. The water content of the different samples was determined at intervals and varied from about 85–90 per cent., that of fresh cabbage varying from 87–90 per cent. of the fresh weight. The results of these experiments are summarised in Table IV and illustrated in the graphs, Fig. 3.

If the results are compared with those in Table II the following rough estimate of the degree of destruction of antiscorbutic material can be made. After one hour's heating at 60° the antiscurvy value of 5 g. cabbage is not greater than that of 1·5 raw cabbage, *i.e.* there is a loss of about 70 per cent. of the original value. Heating for the same time at 70° or 80° reduces the initial value of 5 g. raw cabbage to the equivalent of about 0·5 g. raw, *i.e.* a loss of 90 per cent. has taken place; after heating for one hour at 90° the loss exceeds 90 per cent.

From Table IV it is further evident that 5 g. cabbage heated either for one hour at 60° or for 20 minutes at 90°–100° has a similar effect in just protecting a young guinea-pig from scurvy. We have already seen that a daily ration of 1 g. fresh raw cabbage was the amount determined as necessary for protection from scorbutic symptoms (see p. 424). We may therefore regard 5 g. cabbage cooked for one hour at 60° as about equal in antiscorbutic value to 5 g. cabbage cooked at 100° for 20 minutes and to 1 g. raw cabbage. In other words the rate of destruction of antiscorbutic properties is increased about three times for a rise of temperature of 30° or 40°. If we assume that the destruction of antiscorbutic properties is uniformly accelerated by increase of temperature within this limit, the above results indicate the comparatively low temperature coefficient of about 1·3 for 10° rise of temperature. Assuming this value to be near the truth, then the effect of heating cabbage for one hour at 60° would be equivalent to heating for 46 minutes at 70°, or 35 minutes at 80°, or 28 minutes at 90°, or 21 minutes at 100°: and the experimental results show that the effect of heating 5 g. of cabbage for

Fig. 3. Growth of young guinea-pigs upon a diet of fresh heated cabbage, oats, bran and autoclaved milk (Tables IV, VIII).

——— Curve A. Represents the standard growth of 3 animals upon 30 g. fresh cabbage, oats, bran and water (Expt. 1 Tables II and VII).

Curves B–F. Represent each the average growth of 4 animals upon heated cabbage, oats, bran and autoclaved milk.

– • – • – • Curve B. Cabbage heated to 60° for 1 hour (Expt. 8). —— • —— • Curve D. Cabbage heated to 100° for 20 mins. (Expt. 13).

– – – – – ,, C. ,, ,, 70 ,, ,, (Expt. 9). —— — —— ,, E. ,, ,, 80 ,, 1 hour (Expt. 10).

— • • — • • — ,, F. ,, ,, 90 ,, ,, At † an animal died and the average curve could not be continued (Expt. 11).

• • • • • • ,, G. Citric acid cabbage heated to 90° for 1 hour. At † an animal died and the average curve could not be continued (Expt. 11 A).

At S symptoms first appeared in one or more animals.

one hour at 60° is, in fact, about equal to heating for 20 minutes at 90°–100° (see Table II).

This comparatively small influence of rise in temperature, between the temperatures 60° and 100°, upon the destruction of the antiscurvy value of cabbage is of some interest in connection with the unknown nature of the antiscorbutic factor. It has been suggested that this substance might be a complex protein or an enzyme-like substance. The rate of destruction of most proteins by heat is greatly accelerated with rise of temperature. Egg albumin is coagulated about 635 times faster at 70° than at 60° [Chick and Martin, 1910]; the destruction of emulsin by heat has a temperature coefficient of 7·4 for the same range of temperature [Tamman, 1895], and the action of trypsin is accelerated 5·3 times for every 10° rise in temperature [Bayliss, 1908]. The comparatively insignificant effect of temperature-rise in accelerating the gradual destruction of the antiscurvy substance within the tissues of cabbage leaves, indicates that heat denaturation of a protein-like or of an enzyme-like body is not primarily involved.

In the plant, the antiscorbutic substance is produced and retained in the living cell. On heating at 100° or higher temperatures, killing is almost instantaneous, the collapsed cells becoming permeable alike to water and to the constituents of cell sap. At lower temperatures the killing is more gradual (about 10–15 minutes at 60°) and the tissues remain tough and leathery even after an hour's heating at this temperature. These differences in the behaviour of the tissues on heating may affect the rate of destruction of the antiscorbutic substance. Similar experiments with expressed juices of vegetables and fruits are in progress and may be expected to throw light on this point. According to Holst and Frölich, expressed juice of white cabbage loses nearly all its protective value after heating for 10 minutes at 60°, 70°, or 80°, the expressed juice being, in their opinion, much more thermolabile than when contained in the intact cells. We are at present unable to confirm this statement and in preliminary experiments with expressed juice of turnips a considerable amount of protective value was found to exist after heating for an hour at 80°.

Application of the foregoing Results to Methods of Cooking Vegetables.

The destruction of the antiscorbutic properties of fresh vegetables on heating has an evident bearing on their dietetic value when cooked or preserved. Cooking improves most vegetables by softening their tissues and increasing

TABLE IV. *Experiments with 5 g. rations Cooked Cabbage (+ 60 cc. autoclaved milk).* [For further details see Table VIII.]

Exp.	Cabbage ration		No. of animals	Mean weight		Total gain in wt. %	Duration of exp. in days	Symptoms during life	Post-mortem	Histology of rib-junctions	General conclusions
	Temp. of heating	Time mins		Initial	Final						
8	60° immersed	60	4	339	601	71	88–90	Soreness after 42–60 days	3 normal 1 slightly scorbutic	2 normal 2 incipient scurvy	Protection
9	70° immersed	60	4	334	519	55	90	2 showed no symptoms, 2 developed soreness 42–88 days, 1 showing also "scurvy position"	1 normal 3 fragile bones, and rib-junctions ridged 1 with slight muscular haemorrhage also	2 nearly normal ("incipient scurvy") 2 acute scurvy	Protection Partial protection
10	80° immersed	60	4	347	—	—	34–50, 1 case 90	Soreness after 28–32 days Soreness acute at first, but lessening "scurvy position" in 2 cases	Fragile bones rib-junctions nodular muscular and subcutaneous haemorrhages } in each case	1 nearly normal ("incipient scurvy") 3 acute scurvy	Protection in 1 case, slight protection in 3 cases
11	90° steamed	60	4	334	—	—	21–50	Soreness after 19–35 days, scurvy position, loss in weight	Fragile bones, rib junctions nodular haemorrhages severe	All acute scurvy	No protection
11 A	90° steamed, (citric acid cabbage)	60	4	337	270	−20	26–31	Soreness in 8–22 days	Acute symptoms	All acute scurvy	No protection
12	90° steamed	20	4	324	513	58	90	Soreness after 23–43 days No soreness in 1 case	All normal	All normal	Complete protection
13	100° steamed	20	4	329	485	47	90	3 with no symptoms 1 with slight persistent soreness after 27 days	do.	2 slight scurvy 2 normal	2 nearly protected 2 completely protected
14	100° immersed	20	4	327	493	50	3 lived for 90 (1 died in 30)	1 died in 30 days (pneumonia) with no symptoms: 1 lived 90 days —no symptoms: 2 developed soreness after 23 days	1 normal (after only 30 days) 3 with slight symptoms, brittle jaw, loose teeth, and in 1 case, ridged rib-junctions	1 normal 2 slight and 1 chronic scurvy	1 protected for 90 days 3 partially protected

their digestibility, but it certainly also causes some loss in their antiscorbutic value. This loss is the greater the longer the time of heating, and is very appreciable even when the temperature is far below the boiling point (*e.g.* 60°–70°). In general, slow cooking at a lower temperature is much more deleterious than more rapid cooking at a high one.

At the present time, owing to the shortage of fuel, there has been a great increase in the use of slow cooking of the hay-box type as a substitute for fire or gas cooking, both in private houses and in large communities, *e.g.* hospitals, factories, canteens, municipal kitchens. In this method of cooking, food is given 5–10 minutes' boiling on a stove, and then transferred to a closed box padded sufficiently to prevent much loss of heat by conduction. At least two hours is allowed in the hay box before the food is ready for consumption, and frequently four hours or longer is given, on the theory that in a hay-box it is impossible to over-cook the food. A well-made hay-box should only allow cooling to about 90° in two hours or to about 80° in four hours; but at these temperatures, as has been shown in the preceding section, the rate of destruction of the antiscorbutic factor is not much less than at 100°, although the softening of the tissues takes place much more slowly. It is clear therefore that the longer time needed in the hay-box is a great disadvantage in the case of green vegetables such as cabbage. So far as loss in antiscorbutic value is concerned, the effect of two hours in the hay-box is not much less than that of two hours' boiling.

In cooking green vegetables, it is customary to add a little soda to the water to preserve the green colour. It has been shown experimentally by other workers in this Institute [Harden and Zilva, 1918] that expressed orange juice loses its great antiscorbutic value when made even slightly alkaline shortly before administration. The destruction of the antiscurvy factor by weak alkalis would occur more rapidly at higher temperatures and would enhance the simple destructive effects of high temperature. At the present time, when vegetables form the chief source of antiscorbutic material in the diet of the great majority of the population, the use of soda in cooking should certainly be avoided.

The suggestion has been made[1], that, in order to lessen the inevitable loss of antiscorbutic properties suffered by vegetables on cooking, citric acid to the concentration of 0·5 per cent. should be added to the water in which they are boiled. This suggestion is based on the conclusion emphasised by Holst and Frölich, that fruit juices which are acid are more heat-stable in

[1] Private communication from Major E. D. W. Greig, I.M.S.

respect of the antiscorbutic factor, contained in them, than are the expressed juices of vegetables such as cabbage. According to these workers the juice of lemons and of raspberries retained its antiscurvy properties after heating to 100° for 60 minutes, whereas the freshly expressed juice of cabbage lost its antiscorbutic value after heating to 60°–100° for only 10 minutes. If however the expressed cabbage juice was acidified with 0·5 per cent. citric acid before heating some protection from scurvy was demonstrated. On the other hand expressed heated juice of dandelion leaves was no more protective when acidified, and sorrel leaf juice, which is acid in reaction when expressed, lost all its protective action when heated.

It therefore seemed worth while to investigate this point further and experiments were made on the influence of a weak solution of citric acid upon the antiscorbutic factor in cabbage leaves, both before and after heating. Four animals were fed on a 1·5 g. ration of raw cabbage leaves whose cut ends had 12–24 hours previously been placed in a 1 per cent. solution of citric acid; this passed readily up the vessels into the tissues of the leaf and could be demonstrated there with litmus paper. These four animals were given a ration of 60 cc. autoclaved milk daily and throve as well as those upon a 1·5 g. ration of raw cabbage without any citric acid (Exp. 6 A, Tables II and VII). When an acidified ration of 5 g. was heated to 90° for an hour, however, it had no longer any protective value. Four animals kept on a diet of oats and bran, 60 cc. autoclaved milk and 5 g. acidified heated cabbage died of acute scurvy in 22–30 days. Untreated cabbage heated for this length of time at 90° gave very slight protection, four animals on this ration dying of scurvy in 21–50 days. The addition of the acid therefore had certainly not improved the resistance of the cabbage to the destructive effects of heat[1].

The conclusion therefore to be drawn from these results is that in cooking vegetables it is better to add neither acid nor alkali to the water in which they are boiled.

Influence of Exposure to Temperatures above 100° upon the Antiscorbutic Value of Cabbage.

In order to get evidence as to the effect of heating at higher temperatures it was necessary to use larger rations. With an allowance of 15–30 g. daily, satisfactory nutrition was obtained, without the introduction of autoclaved milk into the diet, even when the cabbage had been heated to 100°–130° for

[1] This result has been confirmed in case of germinated lentils; when boiled for 15 minutes in water containing 0·5 per cent. citric acid, the destruction of antiscorbutic properties was found to be distinctly greater than when boiled for the same period in pure water.

one hour. For example, growth curves of animals fed daily on 15 g. cabbage steamed at 100° for an hour compared favourably with the growth curves of similar animals fed on 15 g. raw cabbage (cf. curve C, fig. 4, with B, fig. 1). Two animals were paired after 60 days of this diet and produced healthy young while still upon this limited cabbage ration.

A summary of the results obtained on heating cabbage to temperatures from 100°–130° as regards loss of antiscorbutic properties is set out in Table V and full protocols of the individual experiments may be found in Table VIII. Curves showing the average growth are shown in Figs. 4 and 5. It will be seen that protection from scurvy was obtained with the 15 g. ration in all cases. Even after heating to 130° for an hour, the symptoms of scurvy in life were only slight and at post-mortem examination the signs consisted of slight ridges upon the rib-junctions in three cases out of four, and small muscle haemorrhages in two cases out of four. All animals in Exps. 15–21, Table V, were in good health after three months upon the specified diets. If, however, slighter manifestations of scurvy were carefully looked for, there is on the whole progressive increase in the degree to which these are apparent, as the temperature at which the rations are heated is raised from 100°–130°. This is indicated by the increase of the number showing slight abnormalities of the histology of the rib-junctions and by gradations from slight tenderness (Expts. 18, 19, cabbage heated at 110° and 120° respectively for one hour) to definite and permanent soreness and swelling of the joints (Expts. 21 and 22, where the cabbage was heated for one and for two hours respectively at 130°).

In Exp. 21 (cabbage heated at 130° for one hour) slight but definite muscular haemorrhages were found beneath the muscles of the knee in two out of four animals after 90 days upon this diet. But not even in this case can the animals be said to have developed well-marked scurvy, and it is clear that there is a large margin of protection against scurvy in the raw 15 g. ration. After heating to 130° for one hour this becomes reduced to the equivalent of rather less than 1·5 g. raw cabbage and greater than 0·5 g. raw cabbage (as may be deduced from a comparison of symptoms with those of Expts. 6 and 7, Table II), and may be estimated at approximately the equivalent of 1 g. raw cabbage. On this basis we arrive at the conclusion that on heating for one hour at 130°, 15 g. of cabbage loses $\frac{14}{15}$, or 93 per cent., of its original antiscurvy properties. In Exp. 19, cabbage heated for one hour at 120°, the five animals were protected from any marked symptoms of scurvy, and the residual antiscorbutic factor may be estimated as, at least, the equi-

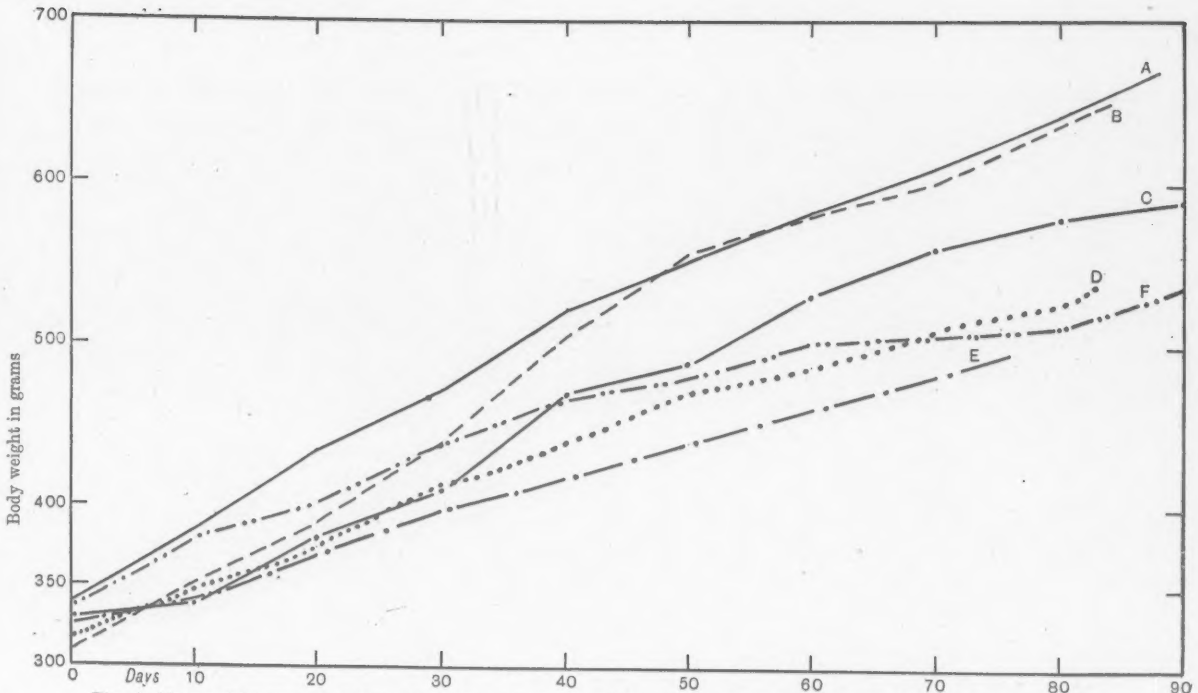

Fig. 4. Mean weight curves of young guinea-pigs on a diet of cabbage cooked 1 hour, oats and bran *ad lib.* and water. Each curve represents the mean weight of all the animals in one experiment calculated from time to time (Tables V and IX).

Curve A. Represents the standard growth curve of 3 animals on a 30 g. ration of raw cabbage (Expt. 1, Tables II and VII).
,, B. Represents the mean growth curve of 3 animals on a 30 g. ration of cabbage cooked at 100° for 1 hour (Expt. 15).
,, C. ,, ,, ,, 4 ,, 15 ,, ,, 100° ,, (Expt. 16).
,, D. ,, ,, ,, 4 ,, 15 ,, ,, 110° ,, (Expt. 18).
,, E. ,, ,, ,, 4 ,, 15 ,, ,, 120° ,, (Expt. 19).
,, F. ,, ,, ,, 4 ,, 15 ,, ,, 130° ,, (Expt. 21).

TABLE V. *Experiments with* 15 *g. cabbage + oats and bran + water.* [For details see Table IX.]

Exp.	Ration	Number of animals	Mean initial weight in g.	Total gain %	Time of exp. in days	Symptoms in life	Post-mortem examination	Histology of rib-junctions	General conclusions
15	30 g. cabbage steamed 1 hr. 100°	3	317	109	90	None	Normal	Normal	Complete protection
16	15 „ „ „ 1 „ „	3	327	82	90	do.	do.	do.	do. do.
17	15 „ „ „ 2 „ „	5	337	56	90	Temporary soreness in 1 case	Normal (fragile bones in 1 case)	do.	do. do.
18	15 „ „ „ 1 „ 110°	5	318	71	85–112	Slight soreness	Normal	Nearly normal or normal	Complete protection (nearly complete protection in 1 case)
19	15 „ „ „ 1 „ 120°	4	326	56	90	Sore knees in 1 case	Jaw fragile in 3 cases	Normal in 2 cases, nearly normal in 2 cases (incipient scurvy)	Complete protection in 2 cases, protection in 2 cases
20	15 „ „ „ 2 „ 120°	4	330	70	90	Soreness in all after 18–26 dys	Bones slightly brittle	3 normal 1 nearly normal	Complete protection in 3 cases, protection in 1 case
21	15 „ „ „ 1 „ 130°	4	346	55	90	Soreness in all after 15–28 dys	Nearly normal, slight haemorrhages in 2 cases	2 normal 2 incipient scurvy	2 complete protection 2 protection
22	15 „ „ „ 2 „ 130°	3	343	12	88	Sore knees in 32 days	Bones fragile, teeth loose	2 definite scurvy	2 partial protection
	(with milk)	1	335	100	86			2 incipient scurvy	2 protection

valent of 1·5 g. raw cabbage, *i.e.* a loss has taken place not exceeding 90 per cent. When heating was continued for a second hour at this temperature the antiscorbutic value was still not reduced below that required for protection. On heating for two hours at 130°, however, definite scurvy symptoms in the form of swollen joints were apparent within 40 days. In this case the 15 g. ration of heated cabbage may be reckoned equivalent to something less than 1 g. but distinctly more than 0·5 g. raw cabbage in antiscurvy value and the loss suffered in this respect may be estimated at more than 93 per cent., and less than 97 per cent.

These rough estimates are collected in Table VI, and for purposes of comparison, the results of the preceding experiments in which the heating was done at temperatures between 60° and 100° are included. It is surprising to find that the destruction at the higher temperatures (Exps. 16–22) is not more complete. Even with the larger ration of 15 g., if the proportional destruction of antiscorbutic factor had proceeded on the same lines as at the temperatures below 100°, we should expect to find the residual amount reduced below the minimum required for protection (the equivalent of about 1 g. raw cabbage) at a temperature much lower than 130°, with one hour's heating. As a matter of fact, definite scorbutic symptoms occurred only when the *15 g. ration was heated for two hours at 130°.*

TABLE VI. *Showing approximately the degree of destruction sustained by the antiscorbutic and growth-promoting properties respectively, of fresh cabbage leaves when exposed to various temperatures.*

				Antiscurvy value		Growth promoting value	
Exp. no	Ration of cabbage, g.	Time of heating, minutes	Temperature C.°	Approximate estimate of heated material expressed in g. of raw cabbage	Loss expressed as % of the original	Approximate estimate of heated material expressed in g. of raw cabbage	Loss expressed as % of the original
8	5	60	60	1·5	70	—	—
9	5	60	70	0·5	90	—	—
10	5	60	80				
11	5	20	90	1·5	70	—	—
12	5	60	90	less than 0·5	more than 90	—	—
13	5	20	100	1·5	70	—	—
16	15	60	100	more than 1·5	less than 90	15	0
17	15	120	100	do.	do.	greater than 5	less than 66
18	15	60	110	do.	do.	—	—
19	15	60	120	do.	do.	—	—
20	15	120	120	more than 1	less than 93	—	—
21	15	60	130	1	93	5	66
22	15	120	130	less than 1 / greater than 0·5	more than 93 / less than 97	0	100

This point is shown perhaps even better by comparing the experiments in which the heating was done at 100° for both larger and smaller ration. Taking Exp. 13 as standard, with a loss of approximately 70 per cent. antiscurvy value after 20 minutes' heating at 100°, and applying this result to Exp. 16, we should expect the antiscurvy factor of the 15 g. ration heated for one hour at 100° to be reduced to the equivalent of 4·5 g. raw cabbage at the end of 20 minutes: to that of 1·4 g. raw cabbage at the end of 40 minutes: and to that of 0·42 g. raw cabbage at the end of 60 minutes. In other words the animals in Exp. 16 should have developed scurvy to a degree similar to that shown by the animals upon a 0·5 g. ration of raw cabbage, see Exp. 7, Table II. As a matter of fact they did nothing of the sort, but remained in excellent health throughout and were enabled to breed satisfactorily upon the diet.

It is therefore not possible to apply, by extrapolation, the results of experiments with a 5 g. cabbage ration to those in which a 15 g. ration is heated at higher temperature or for a longer time. There is no obvious explanation for this discrepancy, and we must conclude that in diets of this type, the *size* of the cabbage ration has some influence upon the degree to which its antiscurvy properties are destroyed by heat. It is evident that with the larger ration some extra source of protection from scurvy is being made available. In searching for this source, another point of difference in the two sets of experiments, is that the source of the fat-soluble growth factor is also different in the two cases. In case of the 5 g. ration, it is mainly derived from the allowance of autoclaved milk; in case of the 15 g. ration, it is derived wholly from the cabbage ration. It is not at once evident in what way this circumstance can affect the antiscorbutic reserves of the diet, and there is much evidence accumulated by the group of workers in this Institute, pointing to a distinct and separate action of these two factors in the nutrition of young guinea-pigs. Miss Tozer has, however, found that a lack of fat-soluble growth factor in the diet is accompanied by a series of changes in the histology of the rib-junction which is almost indistinguishable from that caused by a slight deficiency of antiscorbutic factor (see below, Appendix). It is therefore not impossible, that the antiscorbutic value of a diet may be enhanced and may show greater heat stability, when the antiscurvy factor and the growth fat-soluble factor are derived from the same food stuff. It is noteworthy that scorbutic symptoms supervened in animals fed with the 15 g. cabbage ration which had been heated to 130° for two hours, a point at which, as will be seen below, the reserves of the fat-soluble growth factor

were also exhausted. This factor is believed to be comparatively heat stable and the present investigation affords confirmatory evidence upon this point.

The suggestions contained in the preceding paragraph are put forward with reserve, especially as experiments are now in progress from which it is hoped that more light may be obtained. An investigation is being made on the influence of heat upon the antiscorbutic properties of expressed fruit and vegetable juices, which are deficient in the fat-soluble growth factor. This is supplied by a ration of autoclaved milk, and as the temperature of heating is raised the only change in the diet will be in the allowance of antiscorbutic material provided, the amount and source of the fat-soluble growth factor remaining unchanged throughout. This further investigation should also show whether the inclusion of the antiscorbutic factor in a living tissue has any influence upon its heat stability and the discussion of the whole subject may be profitably postponed until it is completed.

Influence of Heat upon the Growth-promoting Properties of Cabbage.

It has been shown that for full normal growth in a young guinea-pig on a cereal diet, a large daily ration of fresh cabbage (about 30 g.) is necessary. When this is reduced to 15 g. or further to 5 g. daily the resulting growth is also correspondingly diminished (see Table II and Fig. 1). There was no marked difference in appetite amongst the animals of the different experiments for the cereal part of the diet (Table VII), and the diminished growth can best be explained as caused by the lack of sufficient amount of some accessory growth factor in the diet. The work of McCollum and his colleagues, and of Osborne and Mendel has demonstrated that in the case of rats, two accessory growth factors are necessary in the diet if growth is to be made. In our experiments the water soluble growth factor was provided abundantly in the oats and bran ration *ad libitum* [McCollum and Davis, 1915, 1], whilst the fat-soluble growth factor shown to be widely distributed in green leaves [McCollum, Simmonds and Pitz, 1916] was strictly limited. It seems therefore that the limitation of the amount of growth on reducing the cabbage ration is due to the lack of fat-soluble A in the diet. This is in agreement with the general statement of McCollum and Davis that a small amount of the accessory substances is necessary for growth and that "above this amount growth appears to be in some measure proportional to the amount of the accessories present" [McCollum and Davis, 1915, 1]. When the 60 cc. ration

of autoclaved milk was added to the diets containing the small cabbage rations, this deficiency was repaired.

In the results of Expts. 3 and 4, Table II, a diet of 5 g. fresh cabbage with oats and bran and water can be compared with a similar diet to which 60 cc. daily of milk previously autoclaved at 120° for one hour has been added (cp. Fig. 1, curve *C*, and Fig. 2, curve *B*). The animals on the diet which included milk made much better growth than those without it, and their average weight-increase nearly equalled that of animals receiving 15 g. fresh raw cabbage daily and no milk. As regards growth, therefore, cabbage and autoclaved milk can replace each other. In the case of smaller rations of cabbage, *i.e.* of 2·5, 1·5 and especially of 0·5 g. cabbage, in spite of the consumption of the daily ration of autoclaved milk, growth never equalled that upon a 15 g. ration (Fig. 2). In these cases it appears that the reduction of the anti-scorbutic factor may itself have limited growth.

Both growth factors have been regarded as comparatively heat stable. Osborne and Mendel [1915] have shown that the growth promoting powers of butter fat remain unimpaired after heating for two hours at 100°. Other workers in this Institute have drawn attention to the growth promoting effects of milk which has been heated in an autoclave for an hour at 120° [Chick, Hume and Skelton, 1918]. More recently, Steenbock, Boutwell and Kent [1918] working with rats have demonstrated a destruction of the fat-soluble growth factor in butter fat after heating for four hours at 100°. These authors consider that the destructive process is one of low velocity which had not been revealed in the work of other investigators owing to the high initial content of the factor in the original material. These observations do not give any direct evidence as to the rate of destruction of the growth promoting substance, since no systematic comparison of the effects of heated rations has been made with a minimal raw standard ration.

By studying the growth curves of the animals fed on 15 g. cabbage rations heated at various temperatures, it is possible to investigate the influence of temperature on the fat-soluble growth factor.

Comparing C, fig. 4, and B, fig. 1, it is clear that the effect of heating a 15 g. ration of cabbage for one hour at 100° is not accompanied by any change in the amount of growth which was made on this diet, although a 15 g. raw cabbage ration is itself limiting, in that it does not allow maximum growth. A comparison of the slopes of the graphs of Fig. 4 shows that there is a progressive slight decrease in growth rate (10–20 per cent.) as the temperature of heating is raised from 100° to 130°. This can best be explained as the result

TABLE VII. *Experiments with raw cabbage rations.*

Exp.	No.	Sex	Ration of cabbage, g.	Wt of animal, g. Initial	Wt of animal, g. Final	Gain % on total weights	Time of exp. in days	Symptoms	Post-mortem	Histology of rib junctions	Food consumed: Oats, bran, g.	Food consumed: Cabbage, g.	Food consumed: Aut. milk, cc.	General conclusions
1	351	♂♀♂♀	30+water	350	670	91	87	No symptoms	Not examined	Not examined	57	30	—	Complete protection
	352			340	717		92	do.	do.	do.	47	29	—	
	865			330	675		90	do.	do.	do.	41	21	—	do.
	885			340	615		83	do.	do.	do.	61	30	—	do.
2	353	♀♂♀♂	15+water	330	555	74	90	do.	do.	do.	40	15	—	do.
	359			350	580		90	do.	do.	do.	59	15	—	do.
	826			330	620		90	do.	do.	do.	53	15	—	do.
	823			370	650		90	do.	Normal	Normal	55	15	—	do.
3	421	♂	5+water	330	525	26	90	Soreness after 37 days, persistent	Slight haemorrhages; bones slightly brittle	Nearly normal (incipient scurvy)	55	5	—	Protection
	422			320	445		90	No soreness	Normal	do. do.	61	5	—	do.
	425	♂		318	274		90	do.	do.	do. do.	39	5	—	do.
4	420	♀	5+aut. milk	340	530	69	90	Soreness after 44 days, persistent	Slight haemorrhages at knee	do. do.	42	5	55	do.
	423	♀		300	550		90	No symptoms	do. do	do. do.	26	5	57	do.
	424	♀		335	565		90	Soreness after 24 days, persistent	Normal	do. do.	39	5	52	do.
5	440	♂♀♂♀	2·5+aut. milk	320	530	49	90	No symptoms	do.	do. do.	60	2·5	55	do.
	441			302	430		90	Soreness after 26 days, lasting 20 days	do.	do. do.	35	2·5	42	do.
	443			350	440		88	No symptoms	Tibia brittle	do. do.	35	2·5	55	do.
	379			340	515		90	Soreness after 40 days, persistent	Normal	do. do.	56	2·5	45	do.
	380			305	498		90	No symptoms	do.	do. do.	54	2·5	46	do.
6	389	♀♂♀♂♀	1·5+aut. milk	365	502	49	90	Soreness after 35 days, lasting 23 days	do.	Normal	34	1·5	53	do.
	390			330	478		90	No symptoms	do.	do.	48	1·5	45	do.
	391			320	350		70	Loss of weight, no soreness	do.	Slight scurvy	38	1·5	49	do.
	392			355	520		90	Soreness after 38 days, persistent	Jaw fragile	Normal	45	1·5	53	Slight scurvy
	861			355	675		90	Soreness after 33 days, persistent	Normal	do.	46	1·5	59	Protection
	862			325	404		90	Soreness after 87 days, persistent	Femur and tibia brittle; ribs ridged; jaw fragile	Nearly normal (incipient scurvy)	24	1·5	52	do.
6 A	831	♀	1·5 (citric acid) +aut. milk	325	538	79	91	Soreness after 50 days, lasting only 10 days	Normal	Nearly normal (incipient scurvy)	69	1·5	60	do.
	832	♀		372	535		90	Soreness after 50 days, persistent	Jaw fragile, slight haemorrhages	do. do.	28	1·5	60	do.
	833	♂		300	615		90	Soreness after 49 days, lasting 34 days	Normal	Normal	63	1·5	60	Complete protection
	834	♂		322	565		90	Soreness, doubtful throughout	Rib junctions slightly ridged	Nearly normal	34	1·5	59	Protection
7	840	♂	0·5+aut. milk	335	427	18	90	Soreness after 79 days, persistent	Tibia brittle; 5 ribs ridged; slight haemorrhages	Normal	28	0·5	60	do.
	841			330	403		90	Soreness after 30 days, persistent Scurvy position	Tibia and femur brittle; 7 ribs ridged; teeth brittle	Chronic scurvy	25	0·5	58	Chronic scurvy
	847			328	275		67	Swollen knees; loss in weight	Tibia and femur fragile; all ribs ridged; jaw fragile; haemorrhages	Acute scurvy	15	0·5	38	Acute scurvy
	848			330	460		90	Soreness and swollen knees after 26 days, persistent	Four ribs ridged	Slight scurvy	24	0·5	60	Slight scuvry

TABLE VIII. *Experiments with 5 g. heated cabbage rations.*

Exp.	Ration of cabbage, g.	Time of heating, mins.	Temperature °C.	No. of animal	Mean weight of animals, g.		Gain in wt %	Time of exp. in days	Symptoms during life	Results of post-mortem examination	Results of histological examination	Food consumed, g.			General result
					Initial	Final						Oats, bran	Aut. milk	Cabbage	
8	5	60	60	866 ♂	355	615		86	Soreness after 42 dys	Normal	Nearly normal (incipient scurvy)	24	59	5	Protection
				867 ♀	320	568	+71	90	,, ,, 42 ,,	3 rib-junctions ridged, lower molars brittle	do. do.	27	59	5	do.
				868 ♀	320	665		90	,, ,, 60 ,,	Normal	Normal	29	60	5	do.
				869 ♀	360	557		90	,, ,, 43 ,,	do.	do.	34	60	5	do.
9	5	60	70	835 ♀	325	437		91	No symptoms	do.	Nearly normal (incipient scurvy)	44	58	5	do.
				836 ♂	322	428	+55	64	do.	Tibia fragile	do. do.	24	58	5	Protection (cause of death unknown)
				837 ♂	360	790		90	Soreness after 42 dys, scurvy position	3 rib-junctions ridged	Acute scurvy	43	60	5	Definite scurvy
				838 ♀	330	420		91	Soreness after 88 dys	Tibia and jaw fragile; 5 rib-junctions ridged; slight muscular haemorrhages	do.	25	56	5	do.
10	5	60	80	805 ♂	345	395		89	Soreness after 32 dys, with partial recovery	Jaw and teeth brittle; 5 rib-junctions ridged; haemorrhages in muscles	Nearly normal (incipient scurvy)	47	52	5	Partial protection (recovery from scurvy)
				806 ♀	355	356	−8	34	Soreness after 32 dys; scurvy position	All bones very fragile; severe muscular haemorrhages	Acute scurvy	24	52	5	No protection. Death from acute scurvy
				807 ♀	335	270		50	Soreness after 28 dys; scurvy position	do. do.	do.	41	41	5	Slight protection. Death from acute scurvy
				808 ♂	355	263		50	Soreness after 29 dys; scurvy position	do. do.	Definite scurvy	35	44	5	Slight protection. Death from acute scurvy
11	5	60	90 (steamed)	381 ♀	335	240		29	Soreness after 27 dys	do. do.	Acute scurvy	22	41	5	Death from acute scurvy
				382 ♂	350	280	−19	49	,, ,, 35 ,,	do. do.	do.	21	52	5	do do.
				383 ♂	330	275		29	,, ,, 21 ,,	do. do.	do.	20	34	5	do. do.
				384 ♂	320	285		21	,, ,, 19 ,,	do. do.	do.	22	30	5	do. do.
11A	5 (citric acid)	60	90 (steamed)	393 ♀	330	255		26	,, ,, 17 ,,	do. do.	do.	26 (17 dys)	52	4·9 (22 dys)	do. do.
				394 ♂	355	270	−14	31	,, ,, 22 ,,	do. do.	do.	29	46	4·8	do. do.
				395 ♂	340	305		30	,, ,, 21 ,,	do. do.	do.	25	50	5	do. do.
				396 ♂	325	250		26	,, ,, 8 ,,	do. do.	do.	31	51	5	do. do.
12	5	20	90 (steamed)	801	340	510		90	Slight soreness after 30 dys, persistent	Normal	Normal	55	53	5	Complete protection
				802	320	458	+58	90	Slight soreness after 43 dys, persistent	Nearly normal (jaw fragile)	do.	28	54	5	Protection
				803	320	575		90	Slight soreness after 23 dys (temporary)	Normal	do.	34	59	5	Complete protection
				804	315	508		90	No symptoms	do.	do.	36	58	5	do.
13	5	20	100 (steamed)	397 ♀	325	342		90	do.	do.	Definite scurvy (chronic)	35	57	5	Partial protection (chronic scurvy)
				398 ♀	338	520	+48	90	do.	do.	Normal	67	58	5	Complete protection
				399 ♀	330	530		90	do.	do.	Definite scurvy (chronic)	27	49	5	Partial protection (chronic scurvy)
				399a ♂	325	560		90	Slight soreness after 27 dys, persistent	do.	Normal	41	56	5	Protection
14	~ 5	20	100 (immersed)	385 ♀	330	238		28	No symptoms	Emaciated; muscular and subcutaneous haemorrhages	Definite scurvy	33	30	5	Partial protection (died of pneumonia)
				386 ♂	330	578	+31	90	do.	Jaw fragile; 2 rib-junctions ridged	Nearly normal (incipient scurvy)	49	55	7	Protection
				387 ♀	320	360		90	Soreness after 23 dys, acute, but not persistent	Jaw fragile; teeth loose	Acute scurvy (chronic)	29	55	5	Protection (chronic scurvy)
				388 ♀	332	540		90	Soreness after 23 dys, persistent	Jaw fragile	Normal	42	46	5	Protection

TABLE IX. *Experiments with 15 g. heated cabbage rations.*

Exp.	Cabbage ration, g.	Heated		Animal		Wt of animals, g.		Duration of exp. in days	Mean gain %	Symptoms	Post-mortem	Histology of rib-junctions	Food eaten, g.		General conclusion
		Temp. °C.	Time, hours	No.	Sex	Initial	Final						Oats, bran	Cabbage	
15	30	100	1	333	♂	330	760	84	109	No symptoms	Normal	Normal	41	30	Complete protection
				334	♂	305	620	84		do.	Not examined	Not examined	47	30	,, ,,
				335	♂	315	610	84		do.	do.	do.	37	25	,, ,,
16	15	100	1	338	♂	300	598	90	82	do.	Normal	Normal	45	15	,, ,,
				339	♂	330	540	90		do.	Not examined	Not examined	40	15	Produced 1 young after experiment
				340	♂	350	645	90		do.	do.	do.	44	15	
17	15	100	2	354	♂	330	553	90		Soreness, temporarily, after 36 days' experiment	Normal	Normal	45	15	Complete protection
		—		355	♀	330	505	90	56	No symptoms	Not examined	Not examined	32	15	Two young; one born dead
				356	♂	355	465	90		do.	Muscles dark in colour	Normal	41	15	Protection
				357	♂	340	530	90		do.	Tibiae brittle; jaw fragile	do.	39	15	,,
				358	♂	330	560	90		do.	Jaw fragile	do.	35	15	,,
18	15	110	1	365	♂	325	602	90		Temporary soreness for 3 wks after 24th day	Not examined	Not examined	41	15	,,
				366	♀	337	660	90	71	No symptoms	do.	do.	43	15	,,
				367	♀	308	455	86		Soreness, persistent, after 63 dys	Normal	Normal	36	15	,,
				368	♀	296	505	85–112		Soreness, persistent, after 70 dys	do.	do.	46	15	,,
				369	♀	325	498	90		No soreness	Not examined	Not examined	50	15	,,
19	15	120	1	374	♀	315	510	90	56 (excluding No. 378)	Soreness after 56 dys, persistent	Muscles flushed	Nearly normal (incipient scurvy)	45	15	,,
				375	♂	345	517	90		No symptoms	Normal	Normal	37	15	,,
				376	♂	308	507	90		do.	Jaw fragile	do.	47	15	,,
				377	♂	335	515	90		do.	Not examined	Not examined	46	15	,,
				378	♂	335	456	46		Soreness after 16 dys, persistent	Slight muscular haemorrhage	Normal	37	15	,,
20	15	120	2	853	♂	325	622	90	70	,, ,, 33 ,, ,,	Normal	do.	44	15	,,
				854	♀	322	532	87		,, ,, 18 ,, ,,	Two rib-junctions ridged; severe visceral haemorrhage	do.	41	15	,,
				855	♀	325	310	35		Died of diarrhoea	Jaw and tibiae brittle	Nearly normal (incipient scurvy)	28	15	,,
				856	♀	345	515	?		Soreness after 35 dys, persistent	Slight haemorrhages beneath each knee	Normal	44	15	,,
				870	♀	330	575	89		,, ,, 56 ,, ,,		do.	44	15	,,
21	15	130	1	881	♂	349	656	91	55	,, ,, 15 ,, ,, scurvy position	Slight haemorrhages beneath each knee	Normal	47	15	,,
				882	♂	330	515	90		Soreness after 16 dys, persistent, 64 dys, faceache position	5 rib-junctions slightly ridged	do.	38	15	,,
				883	♀	348	522	91		Soreness after 27 dys, persistent	Slight haemorrhages beneath knees. All rib-junctions ridged, 3 with nodules	Nearly normal (incipient scurvy)	44	15	,,
				884		358	450	89		Soreness after 28 dys, persistent, but improving later	Rib-junctions narrowly ridged; molars loose	do. do.	29	15	,,
22	15	130	2	911	♀	330	315	90	12	Soreness after 31 dys, swollen knees	Tibiae brittle, ribs papery, jaw and teeth brittle	Definite scurvy	27	15	Partial protection
				912	♂	350	395	88		Soreness after 34 dys, slightly swollen knees	Ribs ridged, teeth brittle and loose	,, ,,	28	15	,, ,,
				913	♀	350	443	88		Soreness after 20 dys, right knee sore, becoming swollen	Slight haemorrhages	Incipient scurvy	30	15	Protection
				914*	♂	335	670	88	100	Soreness after 31 dys, knees slightly sore, becoming swollen	Knees swollen, molars loose	,, ,,	34	15	,,

* Note 914 received an average ration of 55 cc. aut. milk daily after the 33rd day.

of the destruction of some part of the fat-soluble factor during heating, seeing that the animals were otherwise in good health and there were no definite symptoms of scurvy. This destruction is increased with increase of time (Fig. 5) and at 130° there is a sharp contrast between the mean growth curve of animals fed on the ration heated for one and two hours respectively, the animals on the ration heated for two hours making practically no growth, although remaining very active. One of these animals was given a ration of 60 cc. autoclaved milk daily after the 30th day, and its weight at once rose 66 g. in the next 14 days, and was doubled by the end of the experiment (Table IX), showing that the increase of the fat-soluble factor in the diet caused an immediate access of growth.

By comparing the slopes of the average curves (Figs. 1–4) some idea of the rate of destruction of the fat-soluble growth factor in cabbage leaves at 100°–130° may be obtained. Curves representing the mean growth of experimental animals kept for 90 days on a 15 g. ration given raw (B, Fig. 1) and heated one hour at 100° (C, Fig. 4) are nearly identical. Cooking for an hour has therefore left the growth promoting powers but little impaired. When heating is continued at this temperature for two hours, the resulting growth is much less than that on a 15 g. raw ration but greater than that on a 5 g. raw ration in absence of milk, *i.e.* about half the original amount of growth factor has apparently been destroyed. The mean growth on a ration of 15 g. cabbage heated to 130° for one hour is approximately the same as that of animals on a 5 g. raw cabbage ration; this would indicate a destruction of 60–70 per cent. of the growth factor originally present. After two hours' heating at 130° little or no accessory growth factor remains. On the whole therefore, the fat-soluble growth factor is less sensitive to heat than the antiscorbutic factor. These conclusions are summarised in Table VI.

Summary and General Conclusions.

1. It has been shown experimentally that a very small amount of raw cabbage daily is sufficient to protect from symptoms of scurvy, when added to a diet of grain and autoclaved milk. Histological evidence shows, however, that an apparently healthy animal may be in a condition of incipient scurvy as regards the structure of its growing bones. Possibly these animals might develop symptoms of the disease if kept under less favourable conditions.

2. By a similar method of experiment, determination has been made of the antiscurvy value of cabbage after exposure to various temperatures for

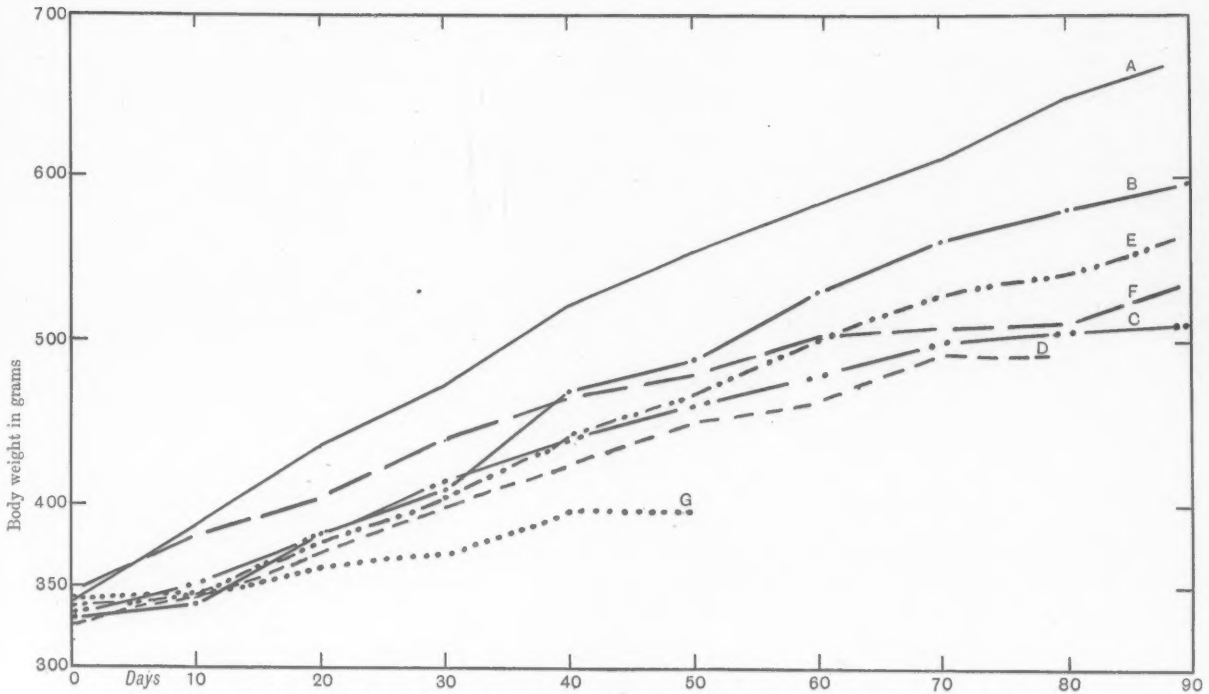

Fig. 5. Comparison of growth of animals fed on oats, bran and water (Tables V and IX), and on 15 g. cabbage heated at 100—130° for varying lengths of time.

Curve A. Represents the standard growth on 30 g. fresh raw cabbage, oats, bran and water (Expt. 1, Tables II and VII).
,, B. Represents the average growth of 4 animals on cabbage heated at 100° for 1 hour (Expt. 16).
,, C. ,, ,, ,, 4 ,, ,, ,, ,, 100° ,, 2 hrs. (,, 17).
,, D. ,, ,, ,, 4 ,, ,, ,, ,, 120° ,, 1 hour (,, 19).
,, E. ,, ,, ,, 4 ,, ,, ,, ,, 120° ,, 2 hrs. (,, 20).
,, F. ,, ,, ,, 4 ,, ,, ,, ,, 130° ,, 1 hour (,, 21).
,, G. ,, ,, ,, 3 ,, ,, ,, ,, 130° ,, 2 hrs. (,, 22).

different intervals of time, and comparison made with that determined for raw cabbage. The antiscurvy factor was found to be exceedingly sensitive to temperatures below 100°. It is estimated that about 70 per cent. of the original value is lost after one hour's heating at 60° and over 90 per cent. after the same period at 90°. After 20 minutes' heating at 90° or 100°, the loss was estimated at about 70 per cent., *i.e.* the same as after 60 minutes at 60°.

3. It follows from the results detailed under (2) that the rate of destruction of the antiscorbutic accessory factor in cabbage leaves is increased only about threefold for an increase in temperature of 30°–40°. This low temperature coefficient is in opposition to the view that heat-denaturation of a protein-like or enzyme-like body is here in question.

4. These facts have some bearing on methods of cooking green vegetables, and indicate broadly that the least loss of antiscorbutic properties will be obtained by cooking green vegetables for a short time at a higher temperature rather than for a longer time at a lower temperature. Other evidence is quoted, which suggests that vegetables should be steamed rather than boiled in water (cp. Expts. 13 and 14, Table IV) and, if boiled, should be boiled in water only, the addition of either acid (citric acid) or alkali (soda) to the water increasing the inevitable loss of antiscorbutic vitamines.

5. Experiments are also described in which autoclaved milk was omitted from the diet and in which larger rations of cabbage were given (15 to 30 g.), heated to temperatures from 100° to 130° for periods of one to two hours. The destruction of antiscurvy properties, though extensive, was found to be much less than was stated by Holst and Frölich and also less complete than would have been expected from the results of experiments in which a smaller ration and lower temperatures were employed. It is suggested that this apparent discrepancy may be in part explained by the fact that in this series of experiments both the antiscorbutic factor and the fat-soluble accessory growth factor are drawn from the same food stuff.

6. The results of experiments described in (5) throw some light on the heat stability of the fat-soluble growth factor present in green cabbage leaves. The effect of heating for 1–2 hours at temperatures from 100°–120° was slight though perceptible. After heating at 130° for two hours, however, a serious amount of destruction had taken place.

7. Further experiments which are in progress with expressed juices of fruits and vegetables given raw and after exposure to different temperatures are expected to throw more light upon the problems dealt with in (5) and (6).

Fig. 1. No. 377. Normal after 3 months on diet of oats, bran, water, and 15 g. cabbage autoclaved 1 hr at 120°

Fig. 4. No. 851. Acute scurvy showing "scurvy position" (foot) (on diet of boiled germinating lentils, unpublished experiment).

Fig. 2. No. 846. Acute scurvy, showing "faceache position" and also position of back limbs (on diet of boiled germinating lentils, unpublished experiment).

Fig. 5. Acute scurvy in animal on purely scorbutic diet of oats, bran and autoclaved milk, showing nearly vertical position of foot.

Fig. 3. No. 394. Acute scurvy showing "faceache position" after 20 days, on diet of 5 g. citric acid cabbage steamed 1 hr at 90°, oats, bran and autoclaved milk.

Fig. 6. No. 510. Showing method of feeding autoclaved milk to the experimental animals.

I am indebted to Dr H. Chick of this Institute for much help in carrying out these experiments, and for valuable suggestion and criticism in their interpretation. I am also indebted to Miss O. Lodge, formerly an assistant in this Department, for her care of the animals used in Expts. 4, 5 and 6; to Miss D. Gardiner for much assistance in the care of many of the experimental animals. I have also to thank my brother, Mr T. F. Delf, for his skill and patience in obtaining the photographs of Plate I.

APPENDIX

By FRANCES MARY TOZER.

THE illustrations (Plate II) are diagrammatic drawings made from histological preparations of the rib-junctions of animals used by Dr Marion Delf in her series of experiments. They are intended to demonstrate the more important histological changes which are observed in animals suffering from scurvy in varying degrees.

The diagnosis made from the histological preparations is, in all cases, independent of Dr Delf's diagnosis of the condition seen during the life of the animal or at the post-mortem examination. The particulars of the experiments were not supplied until the microscope slide was made and reported on, thus eliminating any bias on the part of the observer.

The results as a rule tally closely.

The slight changes observed in the rib-junctions in cases diagnosed as "Nearly Normal" or "Incipient Scurvy" were found to correspond with general good health: in many cases growth was satisfactory, no marked symptoms of scurvy were detected during life and, with very few exceptions, departure from the normal appearance at the post-mortem examination, if any, was insignificant.

The condition found in cases described as "Definite Scurvy" was accompanied by definite symptoms of soreness and a sub-normal condition during life combined with scorbutic lesions on post-mortem examination. The more marked changes in "Acute" cases correspond with severe illness during life and usually death from the disease [see Holst, 1907; Holst and Frölich, 1912; Chick, Hume and Skelton, 1918, p. 137]. These latter authors describe the changes at the rib-junction in the following words:

"Typical changes take place in the costo-chondral junctions. They become swollen, and exhibit a transverse yellow bar.... These appearances correspond with the complete disorganisation of the bone-cartilage junction, and with disappearance of the cartilaginous trabeculae and of the rows of cartilaginous growing cells. In many cases the bone is found to be completely fractured and there is great proliferation of connective tissue across the junction."

This description applies to the "Acute" condition. "Chronic" cases are those which, though suffering from scurvy in varying degrees, are sufficiently protected to prevent death within a period of 90 days: they show, histologically, the condition seen in diagrams 5 and 6. In these cases it seems probable that the ossified band across the junction is an attempt to strengthen the junction in an abnormal manner, the normal process having broken down to an extent depending on the severity of the disease. It is therefore apparent that "Chronic" cases vary in severity according to the degree of protection given.

It is not intended to deal here with the histological changes in the bone cartilage junctions possibly caused by deficiency of the growth, or any other accessory food, factor. In case of the animals to which the following diagrams apply these factors were provided in the diet by oats and bran and autoclaved milk respectively, and it is believed that the amounts consumed were adequate. In other experiments however, where a liberal supply of an antiscorbutic was given and where the fat-soluble A growth factor was known to be deficient, the resulting histological changes in the rib-junctions of the animals examined were found to resemble closely those of "Definite" or of "Definite Chronic" scurvy. In these test cases the antiscorbutic chosen was one believed to be deficient in the fat-soluble A growth factor (for example orange juice). This fact is mentioned because in the case of an animal not receiving an adequate supply of fat-soluble A the resulting changes in the rib-junctions are not dissimilar from, and are likely to be confused with, those caused by scurvy alone.

Note added 13. 1. 19. Since this paper was written further evidence has been obtained, confirming the statement that histological conditions indistinguishable from those termed "incipient" and "definite" scurvy may be caused by an insufficiency in the diet of the fat-soluble growth factor. In cases where the diet consisted of oats, bran and water with a small cabbage ration, it is possible that the histological findings should be thus interpreted (e.g. Table II, exp. 3). Where, however, the cabbage ration, raw or heated, was large (15–30 g, Table V, expts 19 and 21) or where milk was added to the diet (e.g. Table II, expts 5 and 6, Table IV, exp. 8), we have attributed these changes to slight deficiency in the antiscorbutic factor.

Some doubt has, however, arisen as to whether the standard ration of 60 c.c. autoclaved milk may be considered an adequate source of fat-soluble growth factor for guinea-pigs in *all* cases. Further experiments, now in progress, are designed to determine with greater exactness the average amount of fat-soluble growth factor as autoclaved milk necessary for normal growth of guinea-pigs, when all other accessory factors are present in excess.

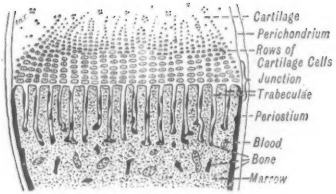

Fig. 1. Normal Rib Junction.

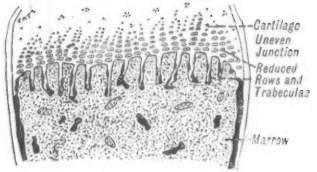

Fig. 2. Nearly Normal Rib Junction, "Incipient Scurvy."

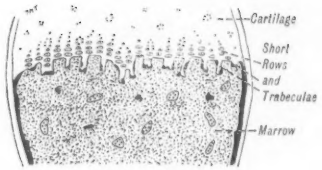

Fig. 3. "Definite Scurvy."

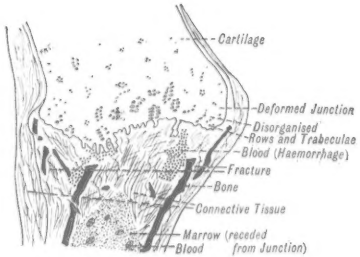

Fig. 4. "Acute Scurvy."

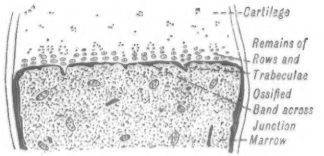

Fig. 5. "Chronic Scurvy" (definite).

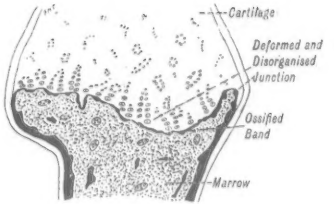

Fig. 6. "Chronic Scurvy" (acute).

DESCRIPTION OF DIAGRAMS, PLATE II.

1. *Diagram of Normal Rib-Junction* as represented by guinea-pig 823 and others.
2. *Diagram of Rib-Junction to illustrate "Incipient Scurvy,"* as represented by guinea-pigs 420–424, 379, 380, 826. This rib-junction is very nearly normal, but shows what may be regarded as the first recognisable signs of the onset of the disease; these are:
   (*a*) An appearance of general abnormality (when compared with the normal).
   (*b*) Unevenness of the junction and slight disorganisation of the rows of cartilage cells.
   (*c*) Shortening of the length of the rows of cartilage cells.
   (*d*) Shortening, and decrease in number of the trabeculae.
   (*e*) Increased amount of blood in the marrow cavity.
3. *Diagram of Rib-Junction to illustrate "Definite Scurvy"* as represented by guinea-pigs 443, 848, 385, 391. Histological symptoms vary considerably in these cases (according to the severity of the disease), and may approach in appearance those characteristic of the "*Acute*" condition. The rows of cartilage cells may be almost normal, but are more often noticeably shortened and usually somewhat disorganised or arranged in circular groups. The trabeculae are usually about half the normal length and have a truncated appearance. The junction as a whole may present an uneven, slightly disorganised appearance and there is sometimes a certain amount of macroscopic deformity.
4. *Diagram of Rib-Junction to illustrate "Acute Scurvy"* as represented by guinea-pigs 381–384, 806–808, 847. In these cases the junction and rows of cartilage cells are often completely disorganised, causing a marked macroscopic deformity of the bone.

   The bone of the shaft is frequently fractured.

   The marrow is no longer in contact with the edge of the junction and the space is filled with a mass of connective tissue; this is very characteristic in cases of fracture. A condition in which the trabeculae have almost disappeared and the rows are much shortened is also found in "Acute Scurvy," in those ribs in which no fracture of the shaft has occurred; usually however there is little or no ossification across the junction.

   There is frequently haemorrhage into the marrow cavity.

Diagrams 5 and 6 illustrate types of "Chronic" scurvy.

5. *May be described as "Chronic Definite Scurvy"* as represented by guinea-pig 841.

   The rows are much shortened, but not disorganised and an ossified band extends across the junction.
6. *May be described as "Acute Chronic Scurvy"* as represented by guinea-pig 387.

   The junction is deformed, and the rows are very disorganised; the trabeculae have disappeared and an ossified band extends across the junction. There is no connective tissue to be seen and no existing, or recent, fracture.

   Particulars of the animals quoted by their numbers in this appendix are given in Tables VI–VIII.

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XXXIV. THE ANTI-SCORBUTIC VALUE OF CABBAGE. II. THE EFFECT OF DRYING ON THE ANTISCORBUTIC AND GROWTH PROMOTING PROPERTIES OF CABBAGE.

By ELLEN MARION DELF and RUTH FILBY SKELTON.

From the Lister Institute of Preventive Medicine.

(*Received November 15th, 1918.*)

In epidemics of scurvy in the past, the attempt has often been made to use dried vegetables or herbs of known antiscorbutic value. Thus in 1720 Kramer, chief surgeon to the Austrian army, in the Austro-Turkish war, was confronted with a serious epidemic of scurvy among the troops; on receiving his appeal for help, the college of physicians of Vienna sent him a great quantity, dried, of the most approved antiscorbutic herbs, but these were of no avail and thousands perished from scurvy [Lind, 1757].

Dried vegetables and herbs were repeatedly used in our own Navy for prevention of scurvy with the same result, and by the middle of the eighteenth century the fact that they could not be relied on for this purpose was well known. Lind [1757, p. 143] says "The latest proposal to the Lords of the Admiralty was a magazine of dried spinage prepared in the manner of dried hay. This was to be moistened and boiled in their food. To which it was objected by a very ingenious physician that no moisture whatever could restore the natural juices of the plant lost by evaporation, and, as he imagined, altered by a fermentation which they underwent in drying." To this a footnote is added, giving the physician's name as Dr Cockburn and stating "The Doctor's judgment is fully confirmed by experience. We find the college of physicians at Vienna sent to Hungary great quantities of the most approved antiscorbutic herbs dried in this manner; which were found to be of no benefit."

This experience was forgotten and more than a century later in the American Civil War, 1861–1865 [*Medical History*, 1888], large rations of dried vegetables allotted to the soldiers, did not prevent outbreaks of scurvy, and

even at the present day, dried vegetables are still regarded by many as equivalent in value to the fresh, in spite of the tragic results of former experience. This opinion would be comparatively harmless, even if translated into action, at a time when a liberal mixed diet was available, but in case of real shortage of the antiscorbutic elements of the diet, it is of obvious importance to know how far reliance may be placed upon dried foods. This is perhaps especially true of the present winter, owing to the unusual failure of the fruit harvest, and to the limited possibilities of importing supplementary supplies.

There is, however, little experimental evidence with regard to the matter. In 1912 a paper by Holst and Frölich [1912] appeared dealing with this question among others. In an exhaustive research upon guinea-pig scurvy, its prevention and cure, these authors used potatoes, carrots, dandelion leaves and cabbage, dried in an incubator for some days at 37° or *in vacuo* at 30°. The material was stored for six months or a year at summer temperature mostly in closed vessels, but in one case in open vessels in an incubator, thus preventing any acquisition of hygroscopic water.

When these vegetables were fed to guinea-pigs in the fresh condition, daily rations of 30 g. added to a dry grain and water diet maintained the animals in health and prevented scurvy. In the case of the dried cabbage, the material was soaked in water for half-an-hour before being fed to the animals, amounts corresponding to the dry weight of 30 g. fresh cabbage being allotted to each animal. Any water unabsorbed was fed to the animals separately. The remaining elements of diet consisted of oats and bran *ad libitum*, and water.

Six animals receiving cabbage dried and stored in the laboratory for one year, died with symptoms of acute scurvy in 21–28 days. On an entirely scorbutic diet consisting of grain and water only, guinea-pigs of the age used in these experiments die of acute scurvy in 20–30 days. It is thus seen that no protection was afforded by the dried cabbage. Another eight animals receiving cabbage dried and stored for six months, died with acute scurvy in 21–37 days; these also received no significant protection from the disease.

A few additional observations were made with the cabbage dried at 37° and stored in an incubator in an open vessel. After six months of such storage a certain amount of protection was afforded by a daily ration equivalent to 30 g. of fresh cabbage to three animals which, however, died ultimately of acute scurvy in 51 to 71 days. Better results were obtained when the cabbage had been stored only six weeks. On this diet two animals died of scurvy after 66 days and two lived for 98 days, one with no apparent symptoms and

the other with evidence of scorbutic lesions on histological examination of the bony tissues.

In a later experiment [Holst and Frölich, 1913] scurvy was prevented in case of two animals for 84 and 62 days respectively with a ration of dried cabbage equivalent to 30 g. fresh; but in this case the cabbage was dried at 37° and kept at that temperature in a desiccator over sulphuric acid. In this case also, as in the other experiments of Holst and Frölich, no increase in weight of the animals took place during the period of experiment.

TABLE I. *Summary of results of Holst and Frölich.*

Ration of Cabbage equivalent to 30 g. fresh cabbage; the rest of the diet consisted of oats, bran and water.

Stored	Age at beginning of experiment	No. of animals	General result
Closed bottles in laboratory	1 year	6	Death from acute scurvy (21–28 days)
do. do.	6 months	8	„ „ (21–37 days)
Open vessel in incubator at 37°	6 „	3	„ „ (51–71 days)
do. do.	6 weeks	4	One lived 98 days, with no apparent symptoms „ „ 98 „ all bones scorbutic Two „ 66 „ all bones scorbutic, also with haemorrhages
Desiccator over sulphuric acid at 37°	4 months	2	One lived 84 days—no symptoms seen in life or at post-mortem One died after 62 days—no symptoms seen in life or at post-mortem

These experiments indicate that the time of storage has a marked effect upon the antiscorbutic value of dried cabbage. This value was greater when the dried material was kept in a very dry atmosphere and prevented from taking up moisture from the air. But even in this case the value is very small in comparison with the fresh material. These workers succeeded in preventing onset of scurvy in guinea-pigs with as small an amount of fresh cabbage as 1 to 2·5 g. daily, and the most successful of their experiments with dried cabbage do not suggest that more than a minimal amount of the original antiscorbutic substance had escaped destruction.

The results obtained by Holst and Frölich with dried cabbage are summarised in Table I. Their experiments with other dried vegetables showed similar deterioration on drying and storing, this being greatest with dandelion leaves and carrots, and least with potatoes. This is in accord with a general opinion held among seamen and travellers as to the practical value of dried potatoes. Instances are not lacking, however, in which their inclusion in a diet has failed to prevent scurvy [*Medical History*, 1888].

First Series of Experiments with Dried Cabbage.

Preliminary experiments with dried cabbage were carried out by one of us (R. F. S.) in the winter of 1916–1917. In July 1916 Dr Harden kindly prepared a stock of material for us dried in a hot room at 37° C. He thought it might be worth while to try the effect of killing the plant cells with boiling water previous to the drying. Such an operation would inactivate any oxidases or other enzymes, present in the cells; these bodies if present, undestroyed, would remain active for a time during the drying process and might be partly responsible for the destruction of the antiscorbutic factor. The results of Holst and Frölich indicate such a possibility, for they achieved a certain amount of protection when the dried cabbage was maintained in an absolutely dry atmosphere, a condition which also would be inimical to any enzyme activities.

Two samples of dried cabbage were therefore prepared; one in which the fresh material was dried in a hot room at 37° forthwith, the other in the preparation of which the fresh material was plunged into boiling water for five minutes previous to drying. Both samples contained about 10 per cent. residual moisture, estimated by drying at 100°. Feeding experiments were started four to six months later, the dried material having been stored meanwhile at laboratory temperature (about 15·5°) in stoppered jars.

Both samples were fed to the animals after moistening with water overnight. The dry weight given was at first the equivalent of 30 g. fresh cabbage, and later double and treble this amount was given. The soaked cabbage was usually eaten well, but when this was not so, a rough estimate was made by eye of the amount left uneaten.

The results of these experiments are shown in tabular form in Tables II and III and illustrated by typical weight curves in Fig. 1. It will be seen by reference to the tables, that both samples of dried cabbage, when offered in amount corresponding to 30 g. fresh cabbage, failed to protect the animals from scurvy. Those on the unboiled sample died with all the symptoms of acute scurvy in 24 to 31 days, showing that they had received no protection. Those on the boiled sample died in a similar manner in 23 to 66 days, some having received distinct protection. These results are in close agreement with those of Holst and Frölich who found no protection in their dried cabbage after six months' storage at laboratory temperature, when no particular precautions were taken as regards access of moisture.

Other animals were offered double and treble the above ration, in the

TABLE II. *Experiments with dried cabbage, unboiled.*

Number of animal	Rations: Dried cabbage offered, g.	Rations: Equivalent in fresh cabbage, g.	Rations: Dried cabbage consumed, g. average	Rations: Equivalent in fresh cabbage, g. average	Rations: Oats and bran consumed, g. average	Weight, g.: Initial	Weight, g.: Final	Weight, g.: ± %	Duration of experiment, days	Symptoms in life	General conclusions
25	3·2	30	3·2	30	—	340	210	−38	31	"Faceache position" on 17th day	Death from severe scurvy on 31st day
26	3·2	30	irregular	—	—	390	230	−41	28	Tender joints on 18th day	Death from severe scurvy on 28th day
27	3·2	30	3·2	30	—	445	270	−39	28	"Faceache position" on 18th day	Death from severe scurvy on 28th day
28	3·2	30	3·2	30	—	265	180	−32	24	Quiet and flabby	In pain, chloroformed on 24th day; post-mortem, severe scurvy
36	6·4	60	6·4	60*	30	350	325	− 7	33	"Faceache position" on 22nd day	In pain, chloroformed on 33rd day; post-mortem, severe scurvy
101	6·4	60	5·0	47*	32	310	215	−31	26	Sore knees on 18th day	Death from severe scurvy
102	6·4	60	5·0	47*	32	295	220	−25	30	Scurvy position and swollen knees on 23rd day	Diarrhoea, chloroformed on 30th day; post-mortem, severe scurvy
98	9·6	90	7·6	71*	32	300	235	−22	25	Tender joints on 12th day	Moribund, chloroformed on 25th day; post-mortem, slight scurvy
99	9·6	90	8·0	75*	28	270	270	0	23	Tender joints on 14th day	Acute attack, fits; chloroformed; post-mortem, slight scurvy
100	9·6	90	9·6	90	31	345	395	+14	61	"Scurvy position" on 28th day	Abscess in thigh, chloroformed; post-mortem, very slight scurvy

* indicates approximate amounts.

TABLE III. *Experiments with dried cabbage, boiled*

Number of animal	Rations: Dried cabbage offered g.	Rations: Equivalent in fresh cabbage, g.	Rations: Dried cabbage consumed, g. average	Rations: Equivalent in fresh cabbage, g. average	Rations: Oats and bran consumed, g. average	Weight, g.: Initial	Weight, g.: Final	Weight, g.: ± %	Duration of experiment, days	Symptoms in life	General conclusions
22	2·6	30	2·6	30	23	390	300	−23	58	"Faceache position" on 22nd day	Leg broken, chloroformed on 58th day; post-mortem, severe scurvy
23	2·6	30	1·3	15*	—	265	180	−32	23	None	Moribund, chloroformed on 23rd day; post-mortem, severe scurvy
33	2·6	30	1·9	21*	26	310	270	−13	34	Tender joints from 21st day	In pain, chloroformed on 34th day; post-mortem, severe scurvy
35	2·6	30	2·6	30	21	320	205	−36	66	"Faceache position" on 20th day, tender joints later	Died on 66th day; chronic scurvy, crippled and undergrown
34	5·2 7·8	60 90	5·2 7·8	60 106 days 90 28 „	42	390	340	−13	134	Knees tender on 34th day	On 127th day gave fresh cabbage, became ill, chloroformed on 134th day; post-mortem, chronic scurvy
96	5·2	60	4·8	55*	29	310	390	+26	66†	Sore joints on 23rd day	Good condition, only slight symptoms in life; chloroformed on 66th day; post-mortem, no scurvy
97	5·2	60	3·8	44*	26	290	345	+19	66†	Sore joints on 14th day	Fair condition; only slight symptoms in life; chloroformed on 66th day; post-mortem, very slight scurvy
93	7·8	90	7·0	80*	44	295	170	−44	34	Sore joints on 26th day	Moribund, chloroformed on 34th day; post-mortem, severe scurvy
94	7·8	90	7·3	84*	30	300	478	+59	66†	"Scurvy position" on 27th day	Splendid condition after recovery from slight attack of scurvy about 27th day; returned to stock
95	7·8	90	7·0	80*	36	325	300	− 8	66†	Tender on 16th day	Good condition but failed to grow; post-mortem, no scurvy

* indicates approximate amounts.
† indicates experiment stopped owing to lack of material for rations.

hope of achieving complete protection. In the case of the double ration of unboiled dried cabbage (*i.e.* equivalent to 60 g. fresh cabbage) death from scurvy resulted in 26 to 33 days, thus again implying little or no protection, but on the double ration of dried boiled cabbage, the result was distinctly better. One animal lived for about 70 days in a condition of chronic scurvy and two others lived for 66 days exhibiting distinct soreness of the joints, the experiment being then terminated by the lack of further supplies of the dried cabbage. A review of the symptoms in life and of the post-mortem appearances shows that these three animals were suffering from scurvy in a more or less acute form. The protection was therefore partial, but was certainly greater than was received by the animals on a similar ration of the unboiled dried cabbage.

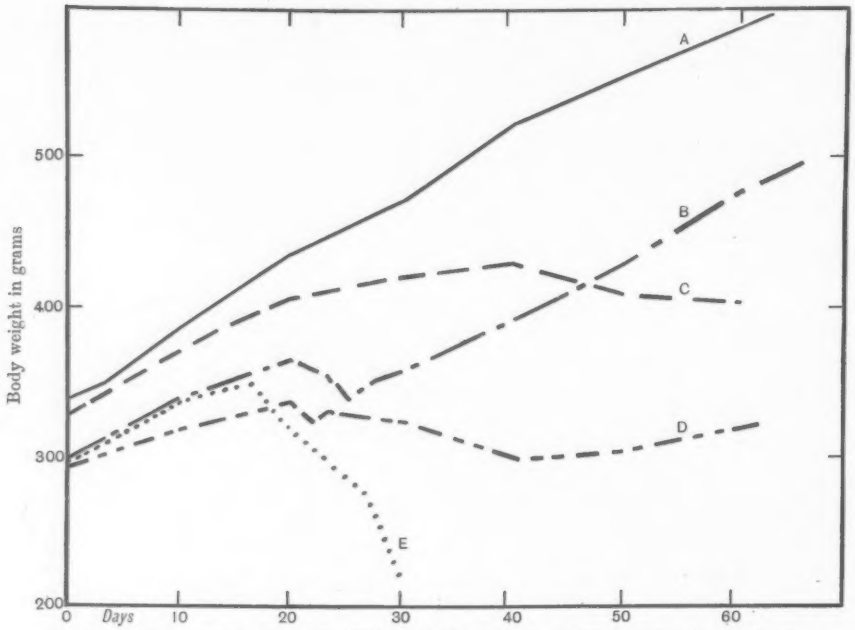

Fig. 1. Comparison of growth on fresh and dried cabbage. Selected curves illustrating preliminary experiments with dried cabbage. The rest of the diet consisted of oats, bran and water ad lib.

——— Curve A represents standard growth on a 30 g. fresh cabbage ration.

—·—·— ,, B represents growth of No. 94, an animal of exceptional vigour, on 7·3 g. dried boiled cabbage (=84 g. fresh).

— — — ,, C represents growth of No. 100 on 9·6 g. dried unboiled cabbage (=90 g. fresh).

—··—··— ,, D represents growth of No. 95 on 7 g. dried boiled cabbage (=80 g. fresh).

····· ,, E represents growth of No. 102 on 5·0 g. dried unboiled cabbage (=47 g. fresh).

On the treble ration (*i.e.* equivalent to 90 g. fresh cabbage) the full amount was not always eaten. Symptoms of scurvy appeared in every case during life, but here again, those on the boiled sample appeared to thrive better than those on the unboiled, and in two cases were in good health upon the 66th day of the diet.

These experiments confirm the general conclusion of Holst and Frölich, that dried cabbage when stored for six months or longer under ordinary conditions of temperature and moisture, offers very little protection if given as the sole antiscorbutic in a guinea-pig's diet. They also indicate that killing the cells by heat before drying is beneficial in lessening the amount of destruction taking place during drying.

This result, taken in conjunction with that of Holst and Frölich, in which dried cabbage was shown to preserve its remnant of antiscorbutic virtue the better if kept in an absolutely dry atmosphere, lends support to the view that some, at least, of the destruction may be due to the activity of an enzyme or other body originally present in the living tissue.

From the results of Holst and Frölich combined with those of control experiments with fresh cabbage made by one of us [Delf, 1918] in connection with another set of experiments we may conclude that a daily ration of about 1 g. fresh cabbage is the minimum amount required to protect a guinea-pig from scurvy upon an otherwise "scurvy diet." In the most favourable cases described above, therefore, we may say that a ration of dried boiled cabbage, equivalent to 60 g. raw cabbage, after four to six months' storage, has a protective value which is less than that of 1 g. fresh raw cabbage, *thus representing a loss of antiscorbutic value of at least* 98 *per cent.*

Experiments with commercially dried cabbage.

In 1917 as part of Food Economy propaganda repeated attempts were made in the daily press to encourage the drying of fresh vegetables. Various articles appeared which implied that the dried material was equivalent in value to the fresh. It therefore seemed desirable to determine more exactly the antiscorbutic and growth promoting properties of fresh and dried cabbage respectively. These experiments were undertaken by one of us [E. M. D.] in 1918, before giving publicity to the results already obtained.

By the courtesy of Mr J. F. Martin, weekly samples of dried cabbage were sent regularly from the Dunnington Farm Produce Factory. These were at first savoys (Feb. 5–April 9, 1918) and later spring cabbages (April

15–June 4, 1918). They were dried without previous treatment, at 120–150° F. (*ca.* 60° C.) for 3–4 hours in an air current driven by electric fan, a process which is essentially that adopted for commercial purposes. The samples were usually received the day following the drying and were stored in stoppered or covered jars in the laboratory at a temperature of 60–65° F. They were dated and used in succession, so that a particular group of animals always received samples of the same age. The ages selected were (*a*) 2–3 weeks, (*b*) 5–6 weeks, and (*c*) 12–14 weeks, in the different series of experiments. The water content varied slightly in the different samples, but averaged about 10 per cent. as before.

Control experiments with raw cabbage leaves.

In a previous communication in this Journal [Delf, 1918] control experiments are described in which young guinea-pigs were kept for a period of 90 days on a diet of oats, bran and varying rations of fresh raw cabbage. The greatest amount of growth was made on a 30 g. daily ration of cabbage (about 90 per cent. increase in body-weight); rather less growth was made on a 15 g. ration (about 70 per cent. increase in body-weight) and still less on a 5 g. ration of cabbage (about 26 per cent. increase in body-weight). Since all these animals were protected from scurvy, it appeared that the limitation of the cabbage ration was reducing too greatly the fat-soluble accessory growth factor known to be present in the cabbage leaves [McCollum and Davies, 1915], the water-soluble growth factor having been abundantly supplied in the cereal part of the diet [McCollum, Simmonds and Pitz, 1917]. Some animals were therefore given a daily ration of milk previously autoclaved for an hour at 120° in addition to a small cabbage ration; 5 g., 2·5 g., 1·5 g. or 0·5 g. cabbage. There is independent evidence that the addition of such a ration of milk improves greatly the nutritive value of the diet without affecting the onset of scurvy [Chick and Hume, 1917; Chick, Hume and Skelton, 1918]. With a diet of 5 g. raw cabbage, oats, bran and 60 cc. autoclaved milk daily, the average growth of the experimental animals was nearly as great as on a diet of oats, bran and 15 g. raw cabbage. With a smaller cabbage ration (2·5, 1·5 g.) the growth was less, but there was no development of scurvy. When the cabbage ration was reduced to 0·5 g. daily, definite symptoms of scurvy were developed in three out of four cases, one animal succumbing to the disease after 50 days. The minimum daily ration of fresh raw cabbage necessary to protect a young guinea-pig from scurvy upon this diet was therefore taken as about 1 g. daily.

TABLE IV. *Experiments w*

Exp.	No. of animal	Sex	Ration	Age of sample, weeks	Weight of animal, g.		Gain % on initial weight	Duration of exp., days	Symptoms	
					Initial	Final				
1	813	♀	3·4 g. dried (= 30 g. fresh)	2–3	312	404	29	90	Lameness from 42nd day	Bones
	814	♂			305	400	31	90	Scurvy position from 27th day	Bones cutan
	815	♂			352	350	0	52	Soreness from 26th day, persistent	Bones haem
	816				350	250	− 29	48	Scurvy position from 31st day	Bones tions
2	824	♂	3·4 g. dried (= 30 g. fresh)	5–6	350	280	− 20	59	Soreness from 31st day	Bones
	825	♀			360	230	− 36	52	,, ,, 23rd ,,	d
	825 A	♀			345	488	+ 41	82	,, ,, 20th ,,	Bones haem
	827	♀			355	210	− 41	48	,, ,, 19th ,,	Bones
	828	♀			325	234	− 28	55	,, ,, 19th ,, (scurvy position)	d
3	842	♀	1·7 g. dried (= 15 g fresh)	2–3	340	475	+ 40	88	Soreness, and scurvy position from 25th day	Bones junct
	843	♂	+ autoclaved milk		330	342	+ 4	80	Soreness, and scurvy position from 24th day	Bones knees
	844	♀			325	530	+ 63	83	Soreness, knees swollen, from 17th day	d
	845	♀			350	480	+ 37	83	Soreness (ribs and knees) from 26th day	Bones
4	877	♀	1·7 g. dried (= 15 g. fresh)	12–14	330	400	+ 21	93	Soreness, scurvy position from 25th day, later very lame	Tibiae severe ridge
	878	♂	+ autoclaved milk		335	225	− 33	56	Soreness, swollen joints, from 17th day	Bones haem
	879	♀			330	225	− 32	52	,, ,, ,, ,, 21st ,,	Bones rhage
	880	♂			350	455	+ 30	90	,, ,, ,, ,, 23rd ,,	Bones cular ridge

ents with Dunnington Farm Dried Cabbage.

Post-mortem	Histology of rib-junctions	Average a consu	
		Oats and bran, g.	cab
Bones somewhat fragile; 9 rib-junctions ridged	Acute scurvy	48	3·4
Bones fragile; haemorrhages in muscles and subcutaneous tissue. 9 rib-junctions ridged	do.	41	3·4
Bones very fragile; severe muscular and subcutaneous haemorrhages; 5 rib-junctions nodular and swollen	do.	45	3·4 3·1
Bones very fragile; severe haemorrhages; all rib-junctions swollen, and nodular	do.	28	3·3 1·7
Bones very fragile; haemorrhages severe	do.	36	3·4 2·7
do. do. do.	do.	23	3·4 2·6
Bones firm, 4 rib-junctions ridged; slight muscular haemorrhage	Nearly normal	23	3·4
Bones very fragile; severe haemorrhages	Acute scurvy	32	3·4
do. do. do.	do.	24	3·4
Bones firm; signs of previous fracture of knees; 3 rib-junctions ridged	Slight scurvy	33	1·7
Bones firm; previous fracture and haemorrhage of knees	do.	18	1·5
do. do. do. do.	do.	30	1·6
Bones firm; previous fracture of knees	Scurvy	31	1·7
Tibiae soft, femur hard; knees severely haemorrhaged; severe subcutaneous haemorrhages; all rib-junctions ridged	do.	14	1·6
Bones very fragile; severe subcutaneous and muscular haemorrhages; all rib-junctions ridged	Acute scurvy	8	1·6 1·1
Bones very fragile; knees fractured; extensive haemorrhages; all rib-junctions ridged	do.	8	1·6 0·8
Bones very fragile; haemorrhages severe (both muscular and subcutaneous). All rib-junctions markedly ridged	Scurvy	13	1·6

Farm Dried Cabbage.

tem	Histology of rib-junctions	Average amount of food consumed daily			Condition	General conclusion
		Oats and bran, g.	Dried cabbage, g.	Aut. milk, cc.		
-junctions ridged	Acute scurvy	48	3·4	—	Thin, but active	Chronic scurvy (killed* after 90 dys)
in muscles and sub-tions ridged	do.	41	3·4	—	do.	do. do. do.
scular and subcutaneous ns nodular and swollen	do.	45	3·4 (26 dys) 3·1 (26 dys)	— —	Wasted	Acute scurvy (died)
emorrhages; all rib-junc-	do.	28	3·3 (31 dys) 1·7 (17 dys)		do.	do.
ages severe	do.	36	3·4 (30 dys) 2·7 (29 dys)		do.	do.
	do.	23	3·4 (22 dys) 2·6 (30 dys)	— —	do.	do.
ridged; slight muscular	Nearly normal	23	3·4 (82 dys)	—	Good	Slight scurvy (killed* after 82 dys)
emorrhages	Acute scurvy	32	3·4 (48 dys)	—	Emaciated	Acute scurvy (died)
	do.	24	3·4 (55 dys)	—	do.	do.
fracture of knees; 3 rib-	Slight scurvy	33	1·7	59	Good	Chronic scurvy (killed* after 88 dys)
re and haemorrhage of	do.	18	1·5–1·6	56	do.	,, ,, 80 ,,
do. do.	do.	30	1·6 1·7	60	do.	,, ,, 83 ,,
of knees	Scurvy	31	1·7	60	do.	,, ,, 83 ,,
severely haemorrhaged; rhages; all rib-junctions	do.	14	1·6 (93 dys)	56	do.	,, ,, 93 ,,
cutaneous and muscular ons ridged	Acute scurvy	8	1·6 (28 dys) 1·1 (28 dys)	56	Emaciated	Acute scurvy (died after 56 dys)
tured; extensive haemor-ged	do.	8	1·6 (28 dys) 0·8 (24 dys)	57	do.	Acute scurvy (died)
ages severe (both mus-l rib-junctions markedly	Scurvy	13	1·6 (90 dys)	58	Good	Scurvy

* By chloroform.

This series of experiments with graded rations of raw cabbage with and without milk [Delf, 1918] forms the basis of the present attempt to determine more precisely the effect of drying on (*a*) the antiscorbutic and (*b*) the growth promoting factors present in the cabbage leaves.

Experiments with Dunnington Farm Greens.

Table IV gives a *résumé* of the experiments made with the Dunnington Farm dried cabbage samples, oats and bran forming the remainder of the diet in all cases.

1. *Dried cabbage 2–3 weeks old (ration equivalent to 30 g. fresh cabbage with oats and bran).* Exp. 1, Table IV.

Four animals fed with the dried cabbage 2–3 weeks old were given a daily ration of 3·4 g., equivalent to 30 g. fresh cabbage. This was readily eaten at first, and, in two cases, throughout the experiment. After 26–42 days all the animals developed scorbutic symptoms and two died (Nos. 815, 816) of acute scurvy in 48 and 52 days respectively. In both these cases, the appetite for the dried cabbage diminished towards the end of the experiment, but in each case not until after the appearance of the first scurvy symptoms (cp. Table IV). This lack of appetite no doubt hastened the last stages of the disease, and was probably caused by the loose and brittle teeth afterwards found at the post-mortem examination. However the disease was definitely recognised in the early stages, before any falling off of appetite had occurred. The two remaining animals survived in fair condition for 90 days when the experiment was terminated. Both post-mortem and histological examination revealed a condition of chronic scurvy, and the animals would probably have survived much longer in a permanently scorbutic condition.

There is here less protection from scurvy than would be expected from a ration of 1 g. fresh cabbage daily, that is to say, the loss of antiscorbutic value due to drying is more than $\frac{29}{30}$ths of that originally present in the fresh state, *i.e.* about 97 per cent.

If we consider the body-weight in the four animals at the beginning and end of the experiment, a net increase of only about 6 per cent. is shown on the total initial weight. It is, however, difficult to generalise from four experiments in which the result as regards growth was so very divergent. In fig. 2 are set out the average growth curves of the animals in Expts. 1–4. Curves B, C, represent the mean growth of the two pairs of animals in Exp. 1. Curve C represents the loss of weight shewn by Nos. 815, 816, which died of

scurvy: in the more favourable cases (Nos. 813 and 814) which survived for the whole three months of the experiment, the total gain in body-weight was about 30 per cent.

This is not much greater than was found with animals receiving a 5 g. ration of fresh raw cabbage, supplemented only by oats, bran and water; in the latter instance the average gain in weight after three months was 26 per cent. of the initial [Delf, 1918]. On this basis we may assume the growth promoting power of 30 g. fresh cabbage, 2–3 weeks after drying, to be reduced to the equivalent of about 5 g. fresh cabbage, *i.e.* about $\frac{5}{6}$ or 86 per cent. of the original, to have been lost. All the animals in Exp. 1, however, developed scurvy and the limitation of growth was probably due to the mutual effect of limited antiscurvy factor, as well as limited fat-soluble growth factor. In a later experiment (Exp. 3) autoclaved milk was added to the diet and the ration of dried cabbage was halved. Scurvy developed in every animal, but the average growth was much better than in Exp. 1. We may therefore conclude that the inferior growth in Exp. 1 must be mainly referred to deficiency in fat-soluble growth factor and that an estimate of 86 per cent. loss through drying and keeping for 2–3 weeks may be approximately correct.

2. *Dried cabbage* 5–6 *weeks old* (*ration equivalent to* 30 *g. fresh cabbage, oats, bran and water*). Exp. 2, Table IV.

Five animals were fed on a ration of 3·4 g., equivalent to 30 g. fresh cabbage, but the dried material had been stored 5–6 weeks. It will be seen from Table IV that symptoms of scurvy developed earlier than in the previous cases, four out of the five dying of acute scurvy. The fifth animal was chloroformed after 82 days when the experiment terminated as the supply of dried material had ended. This animal had exceptional resistance and was only slightly scorbutic when killed. On the whole, however, this set of animals exhibited much less protection than those fed on the samples 2–3 weeks old.

3. *Dried cabbage* 2–3 *weeks old* (*ration equivalent to* 15 *g. fresh cabbage, with oats, bran and autoclaved milk*). Exp. 3, Table IV.

It is clear from the curves B and C (Fig. 2) that on a diet of oats, bran, dried cabbage and water, there is very little growth increment even when the cabbage is recently prepared and the ration equivalent to 30 g. fresh cabbage, and this shows that during drying the growth promoting constituents

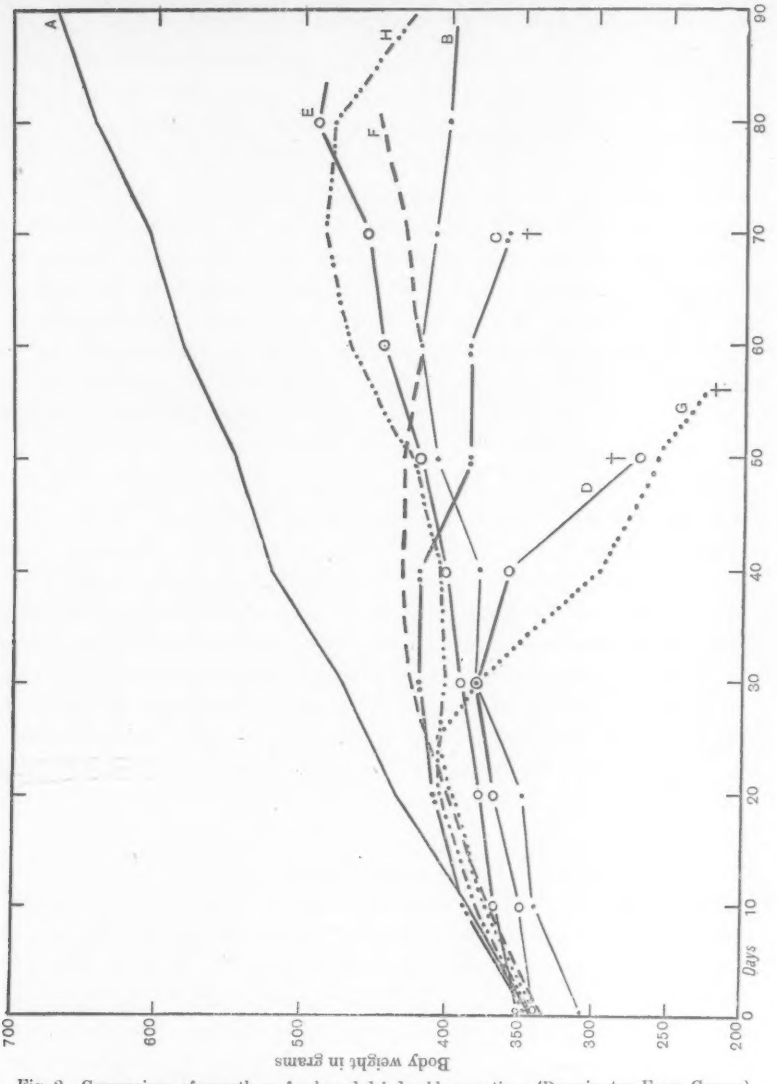

Fig. 2. Comparison of growth on fresh and dried cabbage rations (Dunnington Farm Greens).

——— Curve A represents standard growth on a 30 g. fresh cabbage ration.

Curves B and C represent average growth on diet of 3·4 g. dried cabbage (equivalent to 30 g. fresh) 2–3 weeks old and water.

—•— Curve B represents the mean growth of Nos. 813, 814 in 90 days.

—••— ,, C represents the mean growth of Nos. 815, 816, which both died of scurvy.

Curves D and E represent growth on a diet of 3·4 g. dried (equivalent to 30 g. fresh) cabbage 5–6 weeks old and water.

—○— Curve D represents the mean growth of Nos. 824, 825, 827 and 828.

—○— ,, E represents the mean growth of No. 825 A, an animal of exceptional resistance.

– – – ,, F represents average growth on 1·7 g. dried (equivalent to 15 g. fresh) cabbage 2–3 weeks old with autoclaved milk. This is the mean of Nos. 842, 843, 844, 845.

Curves G and H represent the average growth on 1·7 g. dried (equivalent to 15 g. fresh) cabbage 3 months old with autoclaved milk.

•••••• Curve G represents the mean growth of Nos. 878, 879, both dying of scurvy.

—•••— ,, H represents the mean growth of Nos. 877, 880, both surviving 90 days.

of the fresh leaves have suffered destruction as well as the antiscorbutic factor. In the following experiments, therefore, an attempt is made to distinguish these two effects by increasing the supply of fat-soluble growth factor with a ration of 60 cc. autoclaved milk (120° for one hour).

The results of these experiments proved of extreme interest. The animals made an amount of growth at first nearly equal to that achieved by a ration of 5 g. fresh cabbage with the addition of autoclaved milk (F, Fig. 2). Definite symptoms of scurvy appeared after 17–24 days causing very obvious discomfort. After about three weeks in this condition the animals gradually improved; they became less sore, regained a considerable amount of their former activity and appeared to make an almost complete recovery. At the close of the experiment it was found on post-mortem examination that all had previously suffered from fractured knees and from haemorrhages in the muscles. There is no doubt that the addition of the milk had so much improved the general nutrition of the animals as to ameliorate the severity of the scurvy symptoms.

If we attempt to estimate the amount of antiscorbutic factor left in this ration of dried cabbage, we must place it at less than the equivalent of 1 g. fresh raw cabbage, i.e. *the loss by drying and storing is at least* 93 *per cent.*

This experiment is of interest, in that it shows clearly the effect of a more nourishing diet in protecting from the most severe symptoms of scurvy, in spite of the reduction of the dried cabbage ration to one half the amount which, in the absence of milk, was found to produce death from acute scurvy. This fact is analogous to cases of human scurvy where inferior conditions of diet and comfort have been found to be directly connected with the severity of the attack. Thus when outbreaks of scurvy occurred on board ship, the crew, subjected to greater hardship and to poorer diet than the officers, were commonly attacked with greater severity, even when the amount of fresh food was apparently the same in both cases. It must be admitted, however, that in the greater variety of food enjoyed by the officers, there may also have been found additional traces of the antiscorbutic factor.

4. *Dried cabbage* 12–14 *weeks old* (*ration equivalent to* 15 *g. fresh cabbage, with oats, bran and autoclaved milk*). Exp. 4, Table IV.

Finally, a set of animals was fed on a ration of 1·7 g. dried cabbage 12–14 weeks old (equivalent to 15 g. fresh cabbage) with the addition of autoclaved milk. These were at first particularly strong and healthy animals, but all developed marked symptoms of scurvy in 17–25 days from the beginning of

the experiment. One died of acute scurvy in 52 days, and another in 56 days, the remaining two surviving in a condition of chronic scurvy till the experiment was terminated, at the end of three months. All formed a striking contrast to the animals fed on a similar ration 2–3 weeks old. There is clearly very little protection from scurvy to be gained in this cabbage after three months' storage, however favourable may be the conditions of the animals. The residual antiscorbutic value is indeed less than that provided by a ration of 0·5 g. fresh raw cabbage, representing thus a loss of antiscurvy value equal to 14·5 out of 15, *i.e.* 96–97 *per cent.* The preliminary growth is checked with the appearance of symptoms of scurvy and it seems that lack of the antiscorbutic vitamine is here acting as an inhibiting factor in presence of an ample supply of both essential accessory growth factors in the diet.

From a comparison of the results of Exp. 1 and 2 with those of Exp. 3 and 4, it is clear that the effect of drying fresh cabbage leaves is twofold in destroying the greater part both of the antiscorbutic and of the fat-soluble growth factor. The effect of storage at laboratory temperatures is to cause a further progressive destruction of what antiscorbutic value is left after drying. After three months, the destruction seems to be nearly complete and the product would probably be quite valueless after six months or longer. Probably cold storage would lessen this deterioration, but in view of the great initial deterioration by drying, the value of the product is not worth the considerable expense involved in practice both in drying and in cold storage.

It is important to note that the destruction of the growth factor as also of the antiscorbutic factor, though relatively very great, is not complete. Recent experiments [Hess and Unger, 1918] have been brought forward which prove that hay possesses growth-promoting capacity, and Cohen and Mendel [1918] state that they obtained a "cure" on addition of dried cabbage to the diet of an animal which had developed scurvy on an insufficient ration of fresh milk. With regard to the former statement, we have already noticed this growth-promoting power of hay in the course of some unpublished experiments, but this capacity is doubtless but slight compared with that of the fresh grass from which it is prepared. As it has not proved practicable to obtain control samples of fresh and dried hay of the same plant composition and of known age, no critical experiments on this line have been attempted. With regard to the "cure" reported by Cohen and Mendel on addition of dried greens, the total amount of antiscorbutic in the diet, derived both from the

fresh milk ration and from the dried cabbage (1 g. increasing to 3 g. dry, *i.e.* an amount equivalent to about 10–25 g. fresh), may well have reached the protective value claimed.

Another point of interest is the claim of McCollum and Davis [1915] that the drying of milk does not destroy the growth promoting power of the resulting powder. This result has been confirmed by other workers in this Institute, but it is not yet apparent to what *extent* the great growth promoting powers of fresh milk are being retained. In working with a food so rich in the fat-soluble growth factor, the amount of destruction can only be detected or estimated after careful determinations have been made of some minimum amount which, when given in the fresh and dried state respectively, just allows a selected margin of growth.

Summary and General Conclusions.

1. There is a loss in antiscorbutic properties of more than 93 per cent. when cabbage is dried at a low temperature and stored subsequently for 2–3 weeks at laboratory temperature.

2. After drying and storing for 5–6 weeks at laboratory temperature, a further loss of antiscorbutic properties is suffered.

3. After storage for three months, nearly all the protective value of the fresh material is lost (about 96 or 97 per cent.). The residual amount is distinctly greater if the cabbage is plunged into boiling water before drying.

4. In the drying and storing there is also destruction of the fat-soluble growth promoting accessory factor. This loss has been estimated at about 86 per cent. of the original value after only two weeks' storage.

5. Any remaining antiscorbutic value in dried cabbage is best utilised in the presence of an otherwise nutritious diet such as was afforded by the addition of autoclaved milk to the diet of oats and bran in the above experiments.

6. The preservation of vegetables by drying is an unprofitable process having regard to the labour and fuel involved, to the low nutritive and antiscorbutic value of the product, and to the subsequent deterioration on storage.

In conclusion, our best thanks are due to Mr J. F. Martin of the Dunnington Farm Produce Factory for the trouble taken in dispatching regular weekly supplies of dried cabbage over a period of several months, also to the Ministry of Food, Preserving Section, for kindly making the necessary arrangements. We also wish to acknowledge the help of Miss D. G. Gardiner who has assisted in the tedious work involved in the care and feeding of the experimental animals.

Note.

Since writing this paper, an article has appeared on the "Antiscorbutic Property of Desiccated and Cooked Vegetables" [Givens and Cohen, 1918].

The authors used a basal diet of heated soy bean flour, milk, yeast, paper pulp, sodium chloride and calcium lactate, incorporated into a mixture which was subsequently dried before feeding to the experimental animals (guinea-pigs of various ages). The dried vegetables were cabbage and potatoes, the former being slowly air dried at temperatures of 38–52° ("low" dried) or 75° ("high" dried) or minced and boiled 20 minutes in water before drying. The potato was given after mincing, boiling half-an-hour and drying slowly, on radiators.

The experiments were made with these vegetables given to the extent of about 1 g. daily, in addition to the soy bean diet. The "high" dried cabbage, the cooked dried cabbage and potato were found to be of no antiscorbutic value. The low dried cabbage was found to give partial protection for 59–81 days if consumed at the rate of not less than 1 g. daily. In the most favourable cases life was prolonged for 78 and 81 days respectively, and the post-mortem examination of these animals indicated only slight scurvy: no histological examination of the bones was made.

This result is in accord with our Exp. 3, Table IV, but in the histological examination the scorbutic condition of the animals was still further emphasised.

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XXXV. THE EXTENSION OF THE GAS LAWS TO LIQUIDS AND SOLIDS. I.

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1. *The necessity of re-stating the Gas Laws.*

It was shown by van 't Hoff from Pfeffer's experimental data that in the case of dilute solutions of sugar and other non-electrolytes the osmotic pressure produced by the substance in solution is practically the same as the ordinary outward pressure it would exert if present by itself as gas at the same temperature and in the same concentration per unit volume as in the solution. In other words, if osmotic pressure is taken to represent gas pressure, Boyle's, Charles's or Gay Lussac's and Avogadro's laws for gases can be extended in some sense to substances in dilute solution. Nevertheless there is still no agreement as to how osmotic pressure can represent gas pressure, or how, if it does, the pressure is not in the opposite direction.

A very clear picture of the great divergence of opinion is afforded by the recent discussion on Osmotic Pressure at a meeting of the Faraday Society in May 1917 [Faraday Society, 1917]. Professor Porter defends van 't Hoff's kinetic theory that osmotic pressure is the bombardment pressure of the solute molecules, and therefore, in solutions sufficiently dilute to prevent complicating conditions, has the same value as if the sugar were present as a gas at the same temperature and concentration, with nothing else present in the same space, or only another gas at an ordinary pressure. He points out that the kinetic theory "is the *only one* which directly gives the experimentally ascertained values for dilute solutions." He is forced, however, to make the

assumption that molecules may be treated, not as "real molecules," occupying space, but as "massive points" without extension. Only on this condition does the van 't Hoff theory yield the experimental values for dilute solutions. As was pointed out by other contributors, we cannot, in view of the experimental facts as to highly compressed gases, make any such assumption. Moreover the osmotic pressure on this theory is in the wrong direction. If what is usually called "the osmotic pressure of a solution" were due to extra bombardment pressure caused by the solute molecules, it would be necessary to apply mechanical pressure from the side of the pure solvent in order to neutralise this pressure. Actually the pressure applied in measuring osmotic pressure is pressure on the solution itself—*i.e.* pressure in the *same direction* as the bombardment pressure of the solute molecules.

This latter objection is avoided by the theory, represented in the discussion by Mr Bousfield and other contributors, that osmotic pressure is equivalent to deficiency in the bombardment pressure of solvent molecules in the solution, in consequence of their dilution by the solute. When, however, we endeavour to obtain the experimental results in terms of this theory, the first objection (apart from other objections to the details of Mr Bousfield's theory) applies with the same cogency as to van 't Hoff's theory. The bombardment pressure inside a collection of molecules of the concentration of a liquid amounts to thousands of atmospheres, and the diminution in pressure owing to dilution of the solvent would be enormously greater than the actual osmotic pressure.

It is not necessary to follow at this point the details of the more purely mathematical discussions as to osmotic pressure. To understand the phenomena it is certainly necessary to return first to the fundamental gas laws and the kinetic theory of gases, and consider what must happen when real molecules are present at high concentrations such as exist in liquids.

According to Boyle's and Charles's laws the volume occupied by a given mass of any gas varies inversely as the pressure and directly as the absolute temperature. This is expressed in the equation $PV = rMT$, where P = pressure in atmospheres, V = volume in litres, M = mass in grammes, T = absolute temperature, and r = a constant which varies for the same mass of different gases[1]. When we embody Avogadro's law in the equation it becomes $PV = \frac{22{\cdot}4\,\frac{M}{W}\,T}{273}$ where W = molecular weight of the gas, and 22·4 = volume in

[1] The particular units in which volume, mass, and pressure are expressed have been selected on the score of practical convenience.

litres occupied at 0° C. and 1 atmosphere pressure by the number of grammes of a gas which corresponds to its molecular weight. As $\frac{M}{W}$ = the relative number (n) of molecules (or the number of gramme-molecules) present in different masses of different gases, we can write the equation as $PV = \frac{22{\cdot}4nT}{273}$, or, more simply, $PV = {\cdot}082nT$. When n is constant and equal to 1, ·082 is of course the R of the familiar equation $PV = RT$, when P and V are expressed in the units specified. The kinetic theory of gases has made it possible to deduce this equation from simple principles, and also enables us to realise some of its limitations.

The equation represents very satisfactorily the experimentally ascertained facts in the case of permanent gases or mixtures of them within common limits of pressure and temperature, but, as is well known, breaks down outside these limits, and cannot be extended, except in a very limited sense, to vapours, nor in any clearly intelligible sense, to liquids. The causes of these limitations must now be examined in connection with a proposed re-statement of the equation, so as to permit of its extension to vapours and liquids.

In the first place the molecules of the gas, or gas-mixture, may combine with one another chemically, or may dissociate, on altering the temperature or pressure. It is at once evident that the ordinary gas laws cannot be applied to a change of this character, although they apply, just as before, to the products of the change.

The second limitation of the equation becomes evident even at ordinary temperatures and pressures with gases consisting of large molecules, and at high pressures or low temperatures with all the permanent gases, as, for instance, hydrogen. With T and n constant the product PV sooner or later increases at a growing rate with increase of P; and with P and n constant the quotient $\frac{V}{T}$ increases similarly with diminution of T. This is because, although, on an average, each molecule has an amount of free kinetic energy proportional to its absolute temperature, and therefore keeps clear at each temperature an average intermolecular space inversely proportional to the pressure, the space actually occupied by the molecules themselves in virtue of their relative impenetrability and incompressibility becomes an increasing fraction of the total space occupied by the gas. The volume at absolute zero cannot, therefore, be nothing, as the equation would imply; and with increasing pressure at constant temperature the specific gravity of the gas must increase less and less in proportion to the increase of pressure.

It is clear that the equation could only hold good for an ideal gas, the

molecules of which were points possessed of mass, but having no extension. Ideal molecules of this kind have, of course, been supposed to exist; but, like many other scientific ideals, they do not correspond with actual experience, the supposition that they exist being, for instance, completely at variance with the results of the experiments of Amagat and others on gases at high pressures.

We have thus reached a first point indicating the necessity of re-stating the gas-laws and the fundamental equation which embodies them. This re-statement, as will appear below, is essential for the application of the gas-laws to liquids. We must distinguish the intermolecular volume, or space kept clear by gas-molecules in virtue only of their movements, from the space they would still occupy if they had no movement capable of external transmission. If we call the latter space, in litres, v,[1] we can then, in accordance with the kinetic theory, write the equation of a gas as $P(V - v) = \cdot 082nT$.

When V is constant and $= 1$ litre, v is simply the proportion of occupied space and n is the relative number of molecules or separate particles in a litre, or the molecular concentration. Hence the equation for a gas becomes $P(1 - v) = \cdot 082nT$. But $1 - v$ varies as n at any assigned value of P or of T, either in a pure gas or in any particular gas-mixture. Hence if we call N the molecular concentration or number of gramme-molecules in one litre at $0°$ and a given pressure, the equation $P(1 - v) = \cdot 082NT$ will hold at all temperatures with the same pressure and at all pressures with the same temperature. This form of the general equation is of special convenience in connection with the extension of the gas laws to gases at very high pressure and liquids, and will therefore henceforth be used extensively in this paper, although, as will appear below, with an altered definition of P.

It is only at low pressures, where $1 - v$ is practically equal to 1 that P will vary as N when T is constant. Hence Boyle's law could only be practically true at low pressures and fairly high temperatures, and must become less and less true as the pressure rises or temperature falls. Boyle's law must also deviate more, or less, from truth according to the varying size of the molecule in different gases, and consequent varying value of v at the same

[1] It should be noted that v represents the volume occupied by the molecules when touching one another, and that this is more than the volume of the molecules themselves. In general v corresponds to the b of van der Waals's equation, but since, in the case of each individual gas, the value selected for the b of this equation depends on the value of the $\frac{a}{v^2}$, I have not adopted van der Waals's notation. It will be shown in a further paper that van der Waals's equation is defective.

pressure and temperature. Similarly, it is only when $1 - v$ is practically equal to 1 that with P constant N could vary inversely as T in accordance with Charles's law. Thus at a very high pressure equal volumes of different gases must contain very unequal numbers of molecules, as the experimental results show, and must also expand unequally with the same increments of absolute temperature.

Let us now consider the case of two or more gases present together at very high pressure P or low temperature. Let N_1 be the relative number of molecules and $1 - v_1$ the proportion of free space, or intermolecular volume, in a litre of a gas-mixture at 0° at very high pressure, n the number for a given gas in the mixture, N the number and $1 - v$ the intermolecular volume for the same gas in the pure state at the same pressure, and p its partial pressure in the mixture. It is evident that if the gas were present in the pure state the partial pressure p produced by n molecules would be to the total pressure P as n to N. If, now, without altering the pressure or temperature, we substituted for $N - n$ molecules of the pure gas an equal volume of the other gas or mixture of gases, there could be no change in the partial pressure exercised by the n molecules. But the number of molecules in the gas substituted would, as we have just seen, be different from the number displaced, since at the same temperature and pressure v differs for different gases, so that at very high pressures or low temperatures $1 - v_1$ differs correspondingly from $1 - v$. Hence p is to P, not as n to N_1, but as n to N. This is a matter of great importance, as will appear below, in connection with the application of the gas laws to liquids.

Boyle's law could now be re-stated in the wider and more correct, though less convenient, form that the intermolecular volume of a gas varies inversely as the pressure when the temperature is constant. Charles's law would take the form that at constant pressure the intermolecular volume of a gas varies directly as the absolute temperature. For Avogadro's law the re-statement would be that the number of molecules in a given volume of any gas at the same pressure and temperature varies as the intermolecular volume.

As thus re-stated, and with the proviso that no chemical change occurs in the molecules, the gas laws would represent the experimental facts if it were the case that the molecules simply repelled one another on contact. One can imagine an ideal gas with molecules of this kind; and at ordinary temperatures hydrogen seems to behave in practically the same way as this ideal gas. Actually, however, it is clear from the experimental facts that the molecules, when close together, are powerfully attracted to one another,

so that, once more, the ideal fails to correspond with the reality. This brings us to a third limitation, involving an additional re-statement of the gas laws, but at the same time allowing them to be extended to liquids.

The mutual attractions of the molecules of all gases are clearly shown by the fact that below a certain critical temperature, and with the assistance, if necessary, of a certain increase of pressure, all gases condense to the liquid form. If the gas laws as just restated were correct the formation of liquids and solids would be totally unintelligible, and substances would always behave as they do when high above their critical temperatures. But even when the molecules are, on an average, so far apart that there is no evident condensation, it must happen that the free kinetic energy of a certain proportion of them is for the moment so small that these molecules, in virtue of their mutual attraction, become associated or aggregated together for the moment in a manner analogous to the mutual association of comets or planets in a solar system, though with the molecules constantly colliding with, and not merely revolving round one another. With diminishing temperature or increasing pressure this must tend to happen more and more, and must thus cause a greater and greater deviation from the simple gas laws, even in their amended form: the effect being to diminish the external pressure which would exist but for the molecules becoming associated. When liquid is formed the great majority of the molecules are associated, so that the external pressure is very small.

Although with increasing association together of the molecules of a gas the external pressure exercised by it becomes less than it otherwise would be, yet the mean intermolecular pressure due to the mutual bombardment of the molecules, must remain the same as if no association had occurred, since the temperature, and consequently the mean kinetic energy of the molecules, is the same. Hence the fundamental gas equation still applies if we take P to mean, not external pressure, but the mean intermolecular pressure. When the gas begins to condense to visible liquid (or even, as will be shown later, to solidify) the equation will still hold good. The mean intermolecular pressure is then the "intrinsic" pressure of the liquid plus its vapour pressure, plus any additional external pressure. At lower temperatures the vapour pressure becomes so small in relation to the intrinsic pressure as to be practically negligible.

We must thus again re-state the gas laws by substituting "intermolecular pressure" for "pressure." In the fundamental gas equation $P(1 - v) = \cdot 082NT$ P now stands for intermolecular pressure, and v for the virtual volume of the

molecules in a litre of gas or liquid. We are thus in a position to extend the gas laws to liquids.

In accordance with the molecular conception of matter a liquid is regarded as a system of molecules held together by their mutual attraction, but free to move about within the system except in so far as they collide with one another. They are also kept in constant and very rapid motion by heat. The proportion of free space $(1 - v)$ between the molecules in a litre of the liquid depends on a balance between the attraction of the molecules for one another and the mean free kinetic energy of the molecules. To apply the reasoning on which the kinetic theory of gases is based, this energy will vary, just as with gases, as the absolute temperature T, and will on an average be the same for any sort of molecule or other separate particle present. But since the attractions for one another of the molecules of different liquids vary, the intermolecular pressure, and consequently the free space between the molecules, will also vary in different liquids. The value of $1 - v$ in the fundamental equation is also no longer practically equal to 1, as in gases at ordinary pressures, but is much less than 1. The molecules themselves are also of unequal sizes. Hence equal volumes of different liquids contain very unequal numbers of molecules, and respond with very unequal changes in volume to changes of temperature and pressure. Van der Waals, with the help of his well-known equation, has calculated the value of P for water as about 10,500 atmospheres. If this estimate were correct, and no chemical combination existed among the molecules, the value of $1 - v$ for water would be about 0·118.

When we take P as signifying the intermolecular pressure, and N as the relative number of molecules per litre at 0° and the existing intermolecular pressure of any liquid, we can apply the same equation to liquids as to gases, or to systems of liquid and vapour or gas. If none of the molecules were associated together the external pressure (corresponding to vapour pressure) would be the same as the intermolecular pressure, and the gas would be an ideal or perfect one in the final sense reached in this paper. At temperatures sufficiently above their critical temperatures all the permanent gases approximate to this ideal gaseous state, so that until P becomes so high that $1 - v$ is sensibly less than 1, the gas laws in their crude original form correspond approximately with actual experience. If all the molecules were completely associated together in the liquid form there would be no vapour pressure. At temperatures not far above the melting point but far below the critical temperature this state is approximately realised.

At first sight it might seem that a re-statement of the gas laws in the wider form now given to them is a purely academic procedure, and deprives them of their practical utility, since there are two unknown terms in the re-stated equation, neither of which can be directly measured, though their product is known. It will be seen below, however, that the product of the two unknown terms may be used without either of them being known separately, and that for this reason the equation, in spite of the limitation just referred to, embodies a working hypothesis with very wide and important applications.

2. *Diffusion Pressure and Osmotic Pressure.*

We can now consider the theory of osmotic pressure tentatively suggested by van 't Hoff and accepted by many other writers. This theory has been generally felt, both by chemists and physiologists, to be unsatisfactory, but has become enveloped in a cloud of mathematical reasoning which seems to have hitherto protected it to a considerable extent against attack. Van 't Hoff suggested that the osmotic pressure of a liquid is pressure directly due to bombardment by the solute molecules[1]; and he made the brilliant discovery that for dilute solutions the osmotic pressure corresponds to the external pressure which would be produced if the solute were present as a gas at the existing temperature and in the volume occupied by the solution, with nothing else, or only a gas at ordinary pressure, already occupying this volume.

Now when the solvent (for instance water) is present, the volume is already, as we have just seen, for the most part occupied, and the free space available for the solute molecules is only a small fraction of the total volume. There is thus no intelligible justification for the idea that the addition of the solute molecules would, if they were present under the same conditions as in a gas, add to the existing enormous intermolecular pressure a pressure equal only to what they would produce if the whole space were unoccupied, and they themselves occupied no space. Their actual pressure would be far greater. But even if, in some manner at present quite unintelligible, the intermolecular pressure were increased by an amount just equal to the osmotic pressure, this would give no explanation at all of why pure water should pass *inwards* into the solution through a semi-permeable membrane.

[1] On this subject van 't Hoff (quoted by Lewis [1916]) wrote as follows: "Regarding osmotic pressure as due to a kinetic cause (*i.e.* as produced by impacts of the dissolved molecules [upon the inside of the semi-permeable membrane]), there must exist a proportionality between the number of impacts in unit time and the number of molecules in unit volume. The proof is therefore exactly the same as that for Boyle's Law."

If a gas osmometer were furnished with a membrane permeable to hydrogen, but not to nitrogen, and if there were first of all pure hydrogen at atmospheric pressure both inside and outside the osmometer, and a little nitrogen were then forced in without altering the volume of gas inside the osmometer, the pressure inside would be increased, but there would be no passage of hydrogen inwards, as the partial pressure of the hydrogen inside would not be diminished. It would be just the same in the case of forcing into water a solute to which an ordinary semi-permeable membrane was impermeable. But if the *volume* of gas in the osmometer were increased, even very slightly, at the same time as the nitrogen was added, hydrogen would immediately begin to diffuse inwards, since the partial pressure of the hydrogen in the osmometer would be diminished in proportion to the increase in volume; and inward pressure proportional to this decrease of partial pressure would be developed.

Now when a solute goes into simple solution (without chemical change, such as hydrate formation) the volume of the liquid is always increased, so that the concentration of the solvent per unit volume is diminished. Just as in the case of the gas osmometer, it is this diminution in the concentration of the solvent that matters as regards osmotic pressure. Van 't Hoff's theory of osmotic pressure concentrated attention on the solute molecules, and not on the dilution of the solvent; and this is one reason for the failure of the theory to explain osmotic pressure.

The molecules of a gas are, apart from the almost negligible restraining influence of gravitation, free to move in all directions except in so far as they are stopped or diverted by collisions with other molecules. Now as all the molecules are in free movement, both of translation and rotation, the chances, whatever they may be, of any one molecule in travelling a definite distance in one direction undergoing a collision which will destroy, or partially destroy, its momentum in that direction, must vary as the proportion of occupied space to total available space, *i.e.* in proportion to v. The number of those which escape, or partially escape, will therefore vary as $1 - v$. The number of molecules of any one kind, which, for a given area of surface and unit of time, travel across this distance, must also vary in proportion to the rate of molecular bombardment of the area—*i.e.* in proportion to the total pressure P if only one kind of molecule is present, or to the partial pressure p of the kind in question if other kinds are present. Hence the rate at which molecules of any one kind penetrate through a given layer of the gas must vary as $P(1 - v)$ or $p(1 - v)$. The rate will of course depend also on the

size of the molecules and their mean velocity: also, when the diffusion is through a membrane, on the permeability of the membrane. But when we are only considering relative rates of penetration by the molecules of one particular gas these factors need not be considered, as they are constant. To put this conclusion into other language, *the diffusion rate of any particular gas through a layer of the gas of given surface area and thickness will vary as* $P(1-v)$ *or* $p(1-v)$.

In a gas at ordinary pressure the value of v is so small that it can be neglected where we are comparing variations of diffusion rates with variations of P. Hence in this case diffusion is simply proportional to the pressure P, or for each separate gas to its partial pressure p, where there is a mixture of gases. But in a gas at high pressure the value of v becomes a very appreciable fraction of unity, so that $P(1-v)$ will differ considerably from P. In a liquid such as water the value of $1-v$, as we have already seen, falls to a small fraction of unity. Hence the diffusion rate of water is proportional to only a small fraction of its intermolecular pressure.

We may now consider the case of a gas osmometer with hydrogen at equal but extremely high pressure P inside and outside the membrane, and containing N molecules per litre at 0°. If only pure hydrogen is present on both sides of the membrane the rate of diffusion of hydrogen both inwards and outwards through the molecular layer adjacent to the internal surface of the membrane will be proportional to $P(1-v)$, as we have just seen. But let us suppose that without altering the pressure a mixture containing n molecules of nitrogen to N_1 molecules of hydrogen and nitrogen per litre is substituted for the pure hydrogen in the osmometer and that the mixture is in the perfect gaseous state. In the mixture the value of $1-v$ will be altered to $1-v_1$ and since $\frac{P(1-v)}{P(1-v_1)} = \frac{\cdot 082NT}{\cdot 082N_1T}$, $1-v_1 = \frac{N_1}{N}(1-v)$. Hence the rate of diffusion of hydrogen inwards through the surface layer of molecules inside the osmometer will now be proportional to $\frac{N_1}{N}P(1-v)$ and since, as already pointed out, the partial pressure of any gas is proportional to the ratio of its actual concentration to its concentration in the pure state under the same pressure, the outward rate will be proportional to

$$\frac{N_1-n}{N}P(1-v) = \frac{N_1}{N}P(1-v) - \frac{n}{N}P(1-v).$$

Hence the inward rate is in excess in proportion to $\frac{n}{N}P(1-v)$, and as a direct consequence the inward diffusion pressure is in excess in proportion to $\frac{n}{N}P(1-v)$, since at equal temperatures, in accordance with the kinetic

theory of gases, the pressure in a given direction varies as the number of molecules passing in this direction in unit of time.

But $\frac{n}{N} P (1 - v) = \frac{n}{N} \times \cdot 082NT = \cdot 082nT$. In other words the excess of diffusion pressure inwards owing to the dilution of the hydrogen with nitrogen in the osmometer varies as the outward pressure which the nitrogen would exercise if the molecules occupied no space, so that PV was constant. It is as if PV were constant, whereas it is $P (1 - v)$ that is constant. If the molecules were mere mathematical points repelling one another, so that PV was constant and Avogadro's and Dalton's laws held at all pressures the result would be the same with the same molecular concentration, since intermolecular pressure and diffusion pressure would then be identical. For instance, since PV would be constant and $= \cdot 082\ NT$, P for a gas compressed to the same molecular concentration as water at 0°, would be $\cdot 082 \times 55 \cdot 5 \times 273 = 1243$ atmospheres, instead of van der Waals's calculated value of about 10,500 atmospheres; and $\frac{n}{N} P$ would have the value which $\frac{n}{N} P (1 - v)$ has.

Now the ratio of excess of diffusion pressure inwards corresponds exactly in amount to what van 't Hoff showed to be the osmotic pressure of a very dilute solution; and this points clearly towards the conclusion that osmotic pressure is closely connected with excess of diffusion pressure of the solvent inwards over that outwards, and that the intermolecular pressure is the same in the solution as in the pure solvent, and remains the same with varying temperature, just as in the gas-mixture supposed.

Let us now consider what happens when we equalise the inward and outward diffusion pressure of the hydrogen by raising the pressure of the gas-mixture inside the osmometer, as in the ordinary method of measuring osmotic pressure. Let p_0 be the added pressure needed to equalise the diffusion pressure of hydrogen; and let N_2 be the molecular concentration of the gas-mixture at the increased pressure. The value of $1 - v$ in the gas-mixture will evidently be reduced to $(1 - v) \times \frac{P}{P + p_0}$, and the value of the added pressure of hydrogen, since the ratio $\frac{N_1 - n}{N}$ is not altered by compression, will be $\frac{N_1 - n}{N} p_0$. Hence the equation representing the equilibrium of diffusion pressure will be

$$\frac{N_1 - n}{N} P (1 - v) + \frac{N_1 - n}{N} p_0 = \frac{N_2}{N} P (1 - v) \times \frac{P}{P + p_0}.$$

At the ordinary low pressures of gas-mixtures $1 - v$ equals 1, and $N_2 \times \frac{P}{P + p_0}$ equals N_1, in accordance with Boyle's law. Hence, since N cancels out, the equation becomes $(N_1 - n) P + (N_1 - n) p_0 = N_1 P$. On solving

this equation we find that $p_0 = \frac{n}{N_1 - n} P$. In other words we can equalise the two diffusion pressures of hydrogen by raising the pressure inside the osmometer by a fraction representing the ratio of nitrogen to hydrogen molecules in the mixture. This represents the familiar law of diffusion pressure or osmotic pressure at ordinary gas pressures.

At the other extreme of very high intermolecular pressures, such as those in liquids, the fraction $\frac{P}{P+p_0}$ differs hardly at all from unity until the value of p_0 becomes considerable. Thus if we accept van der Waals's estimate of P for water as about 10,500 atmospheres the value of $\frac{P}{P+p_0}$ will be about 0·998 when p_0 is 30 atmospheres. If the molecules are appreciably compressible[1] $N_2 \times \frac{P}{P+p_0}$ may still be equal to N_1. But even if we consider them as incompressible we can practically simplify the equation into the form

$$(N_1 - n)\, P\,(1 - v) + (N_1 - n)\, p_0 = N_1 P\,(1 - v);$$

and from this it follows that $p_0 = \frac{n}{N_1 - n} P\,(1 - v)$, which, in its turn, is equal to $\frac{n}{N_1 - n} \times \cdot 082NT$. Even, therefore, though we do not know the exact value of P we can still calculate the osmotic pressure of the gas-mixture from its composition if its pressure is extremely high, and the osmotic pressure is very low in comparison.

It is clear also that even if we did not know the molecular concentration of the hydrogen or other "solvent" gas we could still calculate the osmotic pressure if we knew the mass of "solvent" gas in a given volume of the mixture, as compared with the mass in the same volume, and at the same pressure and at 0°, of the pure "solvent" gas, since any error in estimating N would apply equally to the estimate of $N_1 - n$, and would thus cancel out in the equation.

It follows from the equation that if we took P as signifying osmotic pressure and V as volume of "solvent" gas in the mixture, and if n were constant and equal to 1, PV would be equal to $\cdot 082T$, or to RT. If V were taken as volume of the mixture we should have an equation which could be expressed in the form $P\,(V - b) = RT$, since $V - b$ would correspond to $N_1 - n$. Sackur has shown empirically that an equation of the latter form gives the osmotic pressure of sugar solutions; but the b of this equation has evidently nothing to do with the b of van der Waals's equation. If the sugar molecules are hydrates the value of the b is of course increased, as the concentration of free water is diminished.

[1] This question will be discussed in a later paper.

When n is very small relatively to N, it is evident that $\frac{n}{N_1 - n}$ becomes practically equal to $\frac{n}{N}$. Hence at great dilutions van 't Hoff's equation practically gives the osmotic pressure. But as n increases the value of $\frac{n}{N_1 - n}$ diverges more and more from that of $\frac{n}{N}$. Hence van 't Hoff's equation for osmotic pressure gives a value which is increasingly too low, and particularly so if the molecules of the diluent gas are of large size.

If we substitute sugar for nitrogen in the osmometer, and water for hydrogen, the experimental results for osmotic pressure correspond to the values thus deduced theoretically. With very dilute solutions of sugar the results correspond almost exactly with the van 't Hoff equation; but as the strength of the solution increases the van 't Hoff equation gives results which are increasingly too low. Professor Morse and his fellow workers have shown experimentally in their extremely valuable series of researches [1901–1912] that the observed osmotic pressures increase, not in proportion to n, the molecular concentration of the sugar solution, but far more nearly in proportion to the ratio of sugar molecules to weight of water in the solution. This ratio is just the $\frac{n}{N_1 - n} \times N$ of the equation deduced above[1]. The reasoning on which the equation is based thus enables us to predict the experimental results if we assume that the conditions as regards diffusion in the sugar solution are essentially the same as in the very highly compressed gas mixture.

The experimental results for sucrose and dextrose of Morse and his co-workers show that at 0° the osmotic pressure increases with increase of concentration more rapidly than corresponds with the expression

$$\frac{n}{N_1 - n} \times \cdot 082NT.$$

But as is shown in Table I, reproduced from one of their latest papers [1912], they found that (substituting ·0816 for ·082)[2] the accordance with the above expression becomes closer and closer as the temperature rises, and that at 80° the accordance is exact, even in a solution containing as much as 342 grammes of cane sugar per 1000 grammes of water, or about 28 grammes per 100 cc. of solution, this being the strongest solution of which they measured the osmotic pressure.

[1] When $N_1 - n$ is taken as 1000 (the weight in grammes of 1 litre of water at 0°) or when $\frac{n}{N_1 - n}$ is multiplied by N, we obtain Morse's "weight concentration" or n. The n of the present paper corresponds, of course, with van 't Hoff's, and not Morse's n.

[2] Morse took 22·3, instead of 22·4, as the volume in litres occupied by one gramme-molecule of a gas at 0° and 760 mm. pressure. Hence ·0816 is substituted for ·082 in the equation in Table I.

TABLE I. *Osmotic Pressures of Cane Sugar Solutions (Morse and Co-workers).*

Value of $\frac{n}{N_1-n}\times N$, assuming that no hydrates are present. (Weight normal concentration of Morse.)	Ratio of observed osmotic pressure to osmotic pressure calculated from the equation $p_0=\frac{n}{N_1-n}\times 0816NT$ on the assumption that no hydrates are present.											
	0°	5°	10°	15°	20°	25°	30°	40°	50°	60°	70°	80°
0·1	(1·106)	1·082	1·082	1·082	1·084	1·084	1·000	1·003	1·000	1·000	—	—
0·2	1·061	1·063	1·060	1·061	1·062	1·059	1·020	1·011	1·002	1·001	—	—
0·3	1·061	1·058	1·059	1·061	1·060	1·060	1·031	1·024	1·009	0·999	—	—
0·4	1·060	1·059	1·060	1·059	1·060	1·059	1·040	1·038	1·017	1·000	—	—
0·5	1·069	1·067	1·066	1·068	1·067	1·065	1·050	1·046	1·025	1·006	1·000	—
0·6	1·077	1·074	1·073	1·073	1·073	1·071	1·060	1·054	1·032	1·015	1·002	—
0·7	1·083	1·084	1·083	1·083	1·084	1·083	1·069	1·059	1·041	1·020	0·999	—
0·8	1·093	1·093	1·092	1·093	1·093	1·093	1·081	1·067	1·049	1·027	1·008	1·001
0·9	1·104	1·102	1·102	1·102	1·103	1·102	1·089	1·076	1·059	1·033	1·015	1·000
1·0	1·115	1·115	1·113	1·115	1·115	1·113	1·101	1·085	1·071	1·044	1·023	1·000

TABLE II. *Osmotic Pressures of Cane Sugar Solutions at* 0°.

Values of $\frac{n}{N_1-n}\times N$, assuming that no hydrates of sugar are present.	Ratio of observed osmotic pressure to osmotic pressure calculated from the equation $p_0=\frac{n}{N_1-n}\times 0816NT$ on the assumption that no hydrates of sugar are present.		Calculated values of $\frac{n}{N_1-n}\times N$, assuming that the equation $p_0=\frac{n}{N_1-n}\times 0816NT$ is correct. (From Berkeley and Hartley's data.) *
	Berkeley and Hartley	Morse and Co-workers	
·0992	1·008	1·098	·1000
·2978	1·031	1·057	·3070
·6001	1·062	1·075	·6372
·895	1·096	1·099	·9809
·993	1·109	1·110	1·101
2·376	1·278	—	3·037
3·267	1·374	—	4·489
4·126	1·465	—	6·065

* If the values of $\frac{n}{N_1-n}\times N$ are calculated on the assumption that the sugar is present as a pentahydrate the values will be practically the same as in this column, except in the case of the last two values, which will be slightly higher.

Table II embodies the very exact recent determinations of the Earl of Berkeley and E. G. J. Hartley for the osmotic pressures of cane sugar solutions at 0° C. and widely different concentrations, together with the corresponding results of Morse and his co-workers. The table is calculated from Table III in Berkeley and Hartley's paper [1916]. The first column shows the values which $\frac{n}{N_1-n}\times N$ would have if it were the case that no hydrates of sugar were formed, and that all the water in both pure water and sugar solution were present either as simple molecules of H_2O or as the same mixture of simple and more complex molecules of water.

It was suggested by Callendar [1908], who developed a previous suggestion of Poynting, that the reason why a cane-sugar solution at temperatures near 0° does not vary in a proportion nearly equivalent to "weight normal" concentration is that although no sugar hydrates have been isolated, hydrates nevertheless exist in solution. This is undoubtedly the true explanation, as will appear more fully below. On raising the temperature, or increasing the concentration of the sugar solution beyond a certain point, these unstable hydrates would naturally tend to be dissociated. At a temperature of 80° the dissociation seems, from Morse's data, to be complete, so that no sugar hydrates are present.

TABLE III. *Osmotic Pressures of Cane Sugar Solutions at* 0°.

Weight concentration.		Volume concentration.		Osmotic pressure in atmospheres.			
Grammes $C_{12}H_{22}O_{11}$ per 100 g. H_2O	Grammes $C_{12}H_{22}O_{11}+5H_2O$ per 100 g. free H_2O	Grammes $C_{12}H_{22}O_{11}$ per 100 cc.	Gramme-molecules sugar per litre	Actually observed by Berkeley and Hartley	Calculated from the equation $p_0=\frac{n}{N_1-n}\times\cdot082NT$, assuming that the sugar is present as pentahydrate	Calculated from the same equation, assuming that the sugar is not hydrated. (Morse's theory.)	Calculated from the equation $p_0=\cdot082nT$. (Van 't Hoff's theory)
3·393	4·33	3·32	0·097	2·23	2·24	2·22	2·17
10·18	13·21	9·59	0·281	6·85	6·85	6·66	6·29
20·525	27·39	18·26	0·533	14·21	14·17	13·44	11·95
30·61	42·04	25·81	0·754	21·87	21·80	20·04	16·90
33·945	47·07	28·13	0·822	24·55	24·40	22·22	18·41
81·26	130·54	54·24	1·585	67·74	67·66	53·19	35·48
111·73	199·70	66·14	1·933	100·13	103·50	73·14	42·20
141·11	282·97	75·33	2·201	134·84	146·66	92·37	49·31

Table III shows Berkeley and Hartley's values at 0°, as compared with values calculated from the equation $p_0 = \frac{n}{N_1-n} \times \cdot082NT$, on the assumption suggested by Callendar, that the sugar is present as the hydrate $C_{12}H_{22}O_{11} + 5H_2O$, which of course leaves less free water present, and thus increases the value of $\frac{n}{N_1-n}$. It will be seen that the calculated values agree very closely with those observed, until the concentration is so high that there is much more sucrose hydrate than water in the solution. The observed values then begin to fall below those calculated, just as might be predicted as probable on the hydrate theory. From Morse's data it appears that in solutions of lower concentration the whole of the sugar is present as pentahydrate until the temperature rises above 25°. The table also has columns showing the figures calculated on Morse's principle from the equation

$$p_0 = \frac{n}{N_1-n}N \times \cdot082NT$$

on the assumption that the sugar is not hydrated: also the figures calculated

according to van 't Hoff's theory of osmotic pressure from the equation $p_0 = \cdot 082nT$. These columns speak for themselves.

At very low concentrations the values obtained by Berkeley and Hartley diverge from those given by Morse and his co-workers for the same temperature, as shown in Table II; and in all the earlier results, which were obtained at temperatures below 25°, the values of the latter observers at very low concentrations present an anomaly which is very evident in Table I. This anomaly is absent in the results of Berkeley and Hartley at 0°; and it seems almost certain that it is due to a hitherto undetected source of error in the earlier experiments of Morse and his pupils. In these experiments a small proportion of ferrocyanide solution was purposely introduced into the sugar solution and the water, the assumption being that the effect of this on the osmotic pressure would cancel out. But the ferrocyanide, which is ionised and hydrated, must have, on the principles already laid down, the practical effect of increasing the osmotic pressure due to the sugar. Hence the osmotic pressure which would be caused by the sugar solution by itself is lower than the observed osmotic pressure, and in proportion more so at low sugar concentrations. It thus seems possible to account, not only for the general results of osmotic pressure determinations with sugar solutions, but also for the anomalies presented by the early results of Morse and his associates at low temperatures, as compared with the more correct results of Berkeley and Hartley, and of Morse and his associates in their later experiments at higher temperatures.

In so far as we have hitherto considered liquids we have considered solutions as if they were the same as mixtures of perfect gases at very high pressures. But the additional fact must be taken into consideration that the attraction for one another of different kinds of molecules is variable. Thus the different kinds of molecules cannot all come equally close to one another, and the mixture or solution is thus not homogeneous as regards its intermolecular spaces. The attraction of solute molecules for solvent molecules might be either less than, equal to, or greater than the mutual attraction of solvent molecules; and similarly the attraction of solute molecules for one another might be less than, equal to, or greater than their attraction for solvent molecules.

Let us consider the first case. In consequence of the attraction of solute molecules for solvent molecules being less than for solvent molecules to one another the mean clear space round each solute molecule will be greater than round a molecule of solvent. But the result of this will be a surface conden-

sation of solvent molecules round each solute molecule, just as occurs at a free surface. There will thus be local surface tension tending to limit the mean clear spaces round the solute molecules; and the surface condensation will at the same time increase the intermolecular pressure and so diminish the free spaces round the solvent molecules in the area of condensation. Neither in ordinary "crystalloid" nor in "colloid" solutions, nor in gross suspensions, can the distribution of solvent in the space between solute molecules be even, unless solvent and solute molecules have equal attractions for one another.

Now the more the intermolecular pressure round the solute molecules is relatively diminished the more will intermolecular pressure round neighbouring water molecules be increased, provided that a sufficient excess of solvent molecules is present. As a consequence the *mean* intermolecular pressure in pure solvent and in the solution will be practically the same, as will also be the mean clear space per molecule. This would also be the case if the solute molecules had a greater attraction for solvent molecules than solvent molecules have for one another, and likewise if solute molecules had a less or greater attraction for one another than solvent molecules. If solute and solvent molecules had all equal attractions for one another both substances would be in the liquid state: they would be miscible in all proportions; and the intermolecular pressure would be the same at all concentrations. Hence, if P and P_1 be the mean intermolecular pressures in pure solvent and in the solution, P_1 will be equal to P; and if $1 - v$ and $1 - v_1$ be the intermolecular volumes in one litre of pure solvent and of solution, $1 - v_1$ will be to $1 - v$ as N_1 to N (N_1 and N being the numbers of gramme-molecules in one litre of solution and of pure solvent at 0° respectively). The molecules in the two liquids as wholes can thus be treated statistically as if they were homogeneous mixtures of gas molecules at the same intermolecular pressure. In other words, *the intermolecular pressure up to high limits of concentration is the same in pure solvent and solutions in it.* All the experimental data for osmotic pressure and vapour pressure seem to correspond to this conclusion, and the best evidence in favour of it is furnished by this correspondence. The same experimental data show that over a wide range of temperature the intermolecular pressure P remains constant; and further direct experimental evidence on this point will be given in a succeeding paper.

Hence, by the same reasoning as was used in the case of a gas osmometer, the difference between the two diffusion pressures is

$$\frac{n}{N} P (1 - v) = \cdot 082 nT.$$

The osmotic pressure is therefore

$$\frac{n}{N_1 - n} P(1 - v) = \frac{n}{N_1 - n} \times \cdot 082NT,$$

as already shown to be the case from the experimental data. *Osmotic pressure is thus nothing but the excess of pressure required to equalise the diffusion pressure of the solvent from solution to pure solvent with that from pure solvent to solution; and this excess varies as the molecular concentration of the pure solvent, the absolute temperature, and the ratio of solute to solvent molecules.*

It appears that neither van 't Hoff nor succeeding investigators reached any clearly intelligible conception of what osmotic pressure is, though van 't Hoff showed that osmotic pressure is dependent in some way on the gas laws. So long, however, as these laws were embodied in the imperfect equation $PV = \cdot 082nT$, or $PV = RT$, instead of $P(1 - v) = \cdot 082NT$, or $P(V - v) = RT$, the real connection could not be established. Osmotic pressure is commonly referred to as if it were pressure added to a solution in virtue of the presence of the solute, whereas it is simply the increased pressure which must be applied to a solution in order to neutralise the excess in the diffusion pressure of the pure solvent over that of the solvent diluted with the solute. This increased pressure is exactly that required to neutralise the effect of the dilution, just as in the case of a gas mixture at an ordinary pressure; and it is in this indirect way that osmotic pressure depends on the concentration of the solute.

It is evident that with increasing concentration of a solution a point must finally be reached at which, if there are unequal attractions among the molecules, the mean intermolecular pressure begins to alter. Let us consider what must happen in a case not complicated by chemical combination between solute and solvent molecules, or by other chemical changes. The free space round each solute molecule will no longer be controlled by the surface tension resulting from a balancing condensation or rarefaction of solvent molecules round the solute molecule. As a consequence the free spaces round neighbouring solute molecules will finally run together and come to be controlled, not by the mutual affinities of the solvent molecules, but by those of the solute molecules. Unless, therefore, the solute molecules begin to form definite secondary compound molecules they will no longer be "held" in solution by the solvent. In other words the point of saturation will be reached, and the solute molecules can now only exist in the form of aggregates of molecules, whether these aggregates are solid, liquid, or gaseous.

A temporarily supersaturated solution can be prepared by cooling (or

heating) a saturated solution, or, in the case of a gas in solution, by lowering the external pressure of the gas. In this case the solute separates out, taking either the solid form as a precipitate, the liquid form as an emulsion, or the gaseous form as free aggregates of gas. It is evident also from the foregoing account that when two or more solutes are present together in simple solution each solute must diminish the solubility of the others. Hence it is possible to "salt out" one solute by adding another.

When solute molecules combine with water to form hydrate molecules this of course diminishes the concentration of free water molecules present in the solution. As a consequence the rate of diffusion of water from solution to pure solvent is diminished. But as the value $1 - v_1$ for the solution is correspondingly diminished as compared with $1 - v$, there is an equal diminution in the rate of diffusion from pure solvent to solution. The net result is that the excess of diffusion pressure of the pure solvent is the same as if no hydrate had been present. The osmotic pressure is, however, increased, since the value of $N_1 - n$ in the osmotic pressure equation is diminished as compared with what it would be without hydrate formation.

If, on the other hand, the solute molecules are partly or wholly ionised, it is equally clear that excess of diffusion pressure must be increased in proportion to the ionisation, provided that the membrane is impermeable to the ions. That increase of osmotic pressure, proportional to the ionisation in the case of very dilute solutions, is actually present was pointed out by Arrhenius. It seems probable that the process of ionisation in an aqueous solution, as was suggested by van der Waals, is a tearing apart of solute molecules owing to the affinity of their constituent atoms or atomic groups for water molecules, with formation of unstable hydrate molecules which cannot pass through the semi-permeable membrane. This hypothesis gets rid of the great difficulties associated with the assumption that ions are free atoms or completely unsaturated groups of atoms, and at the same time renders intelligible the influence of ionisation on osmotic pressure, the absence of any considerable absorption of heat in the process of ionisation, the relations between ionisation and dilution of a solution, and the action of water as a catalyst. The hydrate formation will add to the increase of osmotic pressure due to the mere splitting up of solute molecules in ionisation.

In the case of water, and to a less extent in that of other solvents, there is clear evidence that the molecules are to a large extent chemically combined with one another. This is shown, for instance, by the fact that as the temperature falls the contraction of water becomes less and less for each degree of

fall, quite out of proportion to the foregoing re-statement of Charles's law, and that below 4° there is an actual expansion. In this respect water presents a striking contrast to such liquids as mercury or alcohol. A similar anomaly appears in the compressibility of water. These anomalies correspond in direction with the expansion of about 10 per cent. on solidification to ice, so that when molecules of water unite with one another their virtual volume is increased.

Water must evidently be regarded as a solution of compound water molecules in simple H_2O molecules. In the solution the mean intermolecular pressure will therefore, in accordance with the principles already laid down, be the same as if no solute were present. But since the compound solute molecules are readily dissociable into simple H_2O molecules, they will be dissociated instantly in proportion to any fall in the diffusion pressure of the simple H_2O molecules owing to their dilution by an ordinary solute. Thus the compound H_2O molecules will reduce the fall which, but for their presence, would have occurred in the diffusion pressure of the H_2O molecules, and this reducing influence will be proportional to the concentration of the compound molecules. In other words, we must, in estimating the reduction of the diffusion pressure of the simple H_2O molecules, calculate, not from the original concentration of simple H_2O molecules, but from the original total concentration of H_2O molecules, combined or not combined with one another. We must therefore calculate from the "weight concentration" in Morse's sense, allowing only for any water which is present in relatively stable combination with the solute molecules. In calculating osmotic pressure we can thus neglect altogether the fact that the molecules of solvent are to a large extent combined with one another. Hence if we call M, M_1, m, the masses of pure solvent, solution and solute in one litre at 0°, we can write the osmotic pressure equation as $p_0 = \frac{nM}{M_1 - m} \times \cdot 082T$.

The same considerations are valid also in the case of the osmotic pressure of gases at very high pressures. The molecules of the "solvent" gas may be to a large extent aggregated together, and this of course diminishes the external pressure which the same mass would otherwise exert; but in calculating osmotic pressures we have only to take account of the total concentrations of molecules.

In connection with osmotic pressure much confusion has arisen through failure to distinguish between the conditions which determine the amount of osmotic pressure and those which determine whether or not a membrane is permeable to a solute. This confusion occurs more particularly in connection

with the theory of I. Traube as to the cause of osmotic pressure, and its relation to the surface tensions of solutions and the intermolecular pressure. His data show an evident relation between lowering of surface tension of a solution and permeability of membranes by the solute, but none between surface tension of solutions and amount of osmotic pressure. It seems clear that the surface tension of a solution (as distinguished from a pure liquid) has no direct relation to its intermolecular pressure. Traube concluded that osmosis is due to the fact that a liquid of high intermolecular pressure absorbs, *ipso facto*, liquids of lower intermolecular pressure. This theory is inconsistent with the principles embodied in the kinetic theory of fluids.

The theory that osmotic pressure is essentially dependent on the existence of chemical combination between solvent and solute molecules has now almost ceased to be supported. It is perhaps, therefore, scarcely necessary to point out that this theory fails altogether to explain the connection between osmotic pressure and the gas laws. Of course molecular attraction plays an essential part in the explanation, but only in so far as it determines solubility.

3. *Vapour Pressures of Solutions.*

The vapour pressure of a substance is the pressure of its vapour at which inward diffusion balances the outward diffusion consisting in the flying off of molecules from the surface. In accordance with the conception of a liquid as a system of freely moving molecules held in proximity to one another by molecular attraction, and subject to the fundamental gas laws, the escape of molecules from the surface is due to the kinetic energy of a certain proportion of them being so great that they can fly off, thus producing external pressure. This proportion must vary inversely as the excess of kinetic energy required to overcome the molecular attraction which holds them; and this energy, as pointed out by van 't Hoff, will vary as the latent heat of vaporisation. The proportion must also depend on the number of molecules per unit of free surface, and on the absolute temperature, since this determines the mean energy of molecular movement.

Since anywhere near ordinary atmospheric pressure $1 - v$ is practically the same in a gas as 1, at these pressures the rate of escape of molecules from the surface will vary with the available excess of intermolecular over "intrinsic" pressure, there being in this reference no appreciable hindrance from the atmosphere. In accordance with the principles laid down in connection with osmotic pressure the rate of passage of vapour molecules from the atmosphere into the liquid must, other things being equal, vary as the

pressure of the vapour molecules and as the value of $1 - v$ for the particular liquid, just as in the case of liquid diffusion. Hence if we call the vapour pressure p, the inward diffusion pressure which balances the outward is $p(1 - v)$, and not p. The diffusion pressure of a vapour or gas into a liquid is thus only a fraction of the total vapour or gas pressure, just as the diffusion pressure of one liquid into another is only a fraction of its intermolecular pressure.

If, now, a solvent is diluted by the presence of a solute with no appreciable vapour pressure, and the temperature and intrinsic pressure are unaltered, the rate of passage, and consequently the pressure, of vapour molecules outwards will be diminished in the proportion of $\frac{N_1 - n}{N}$, where n, N, and N_1 denote relative number of molecules per litre of solute, pure solvent, and solution. As already seen, the values of $1 - v$ for the pure solvent, and $1 - v_1$ for the solution vary as N to N_1 so long as the intermolecular pressure is the same in pure solvent and solution. Hence if p be the vapour pressure of the pure solvent the diffusion pressure of the solvent outwards from the liquid will be diminished to

$$\frac{N_1 - n}{N} p(1 - v) = \frac{N_1}{N} p(1 - v) - \frac{n}{N} p(1 - v);$$

and the pressure inwards will be altered to $\frac{N_1}{N} p(1 - v)$. Hence the diffusion pressure outwards will be less by $\frac{n}{N} p(1 - v)$ than the previous inward pressure. The new vapour pressure will therefore be lower, and if the diminution of vapour pressure be designated p_d, $\frac{N_1 - n}{N} p(1 - v)$ must be equal to $\frac{N_1}{N}(p - p_d)(1 - v)$. From this equation it follows that $\frac{n}{N} p = \frac{N_1}{N} p_d$, so that $p_d = \frac{n}{N_1} p$. In other words, at equal temperatures *the depression of vapour pressure of the solvent is proportional to the ratio between concentration of solute molecules and of total molecules in the solution.*

Now this is the proportion discovered experimentally by Raoult. At great dilutions $\frac{n}{N_1}$ is practically equal to $\frac{n}{N}$, the proportion given in van 't Hoff's equation; but both the above theoretical deduction and the actual experimental results (as will be shown below) indicate that Raoult's equation is the correct one.

By similar reasoning it can be shown that if the vapour pressure of a solute in a given solution is p_1, its vapour pressure in stronger or weaker solutions will vary as $\frac{n}{N_1}$.

It has been shown above that within the limits up to which osmotic

pressures have hitherto been measured, the osmotic pressure increases in proportion to $\frac{n}{N_1-n}$, in accordance with the experimental results. But so long as n is small in relation to N_1, as is the case with all existing measurements of osmotic pressure, the difference between $\frac{n}{N_1}$ and $\frac{n}{N_1-n}$ is small, so that increase of observed osmotic pressure with increase of concentration of solute must run almost parallel with diminution of vapour pressure.

The same factors, such as combination among solute and solvent molecules (as in hydration), or ionisation, which cause increases in osmotic pressure will tell in the same direction, and, up to moderate concentrations, to nearly the same extent, in diminishing vapour pressure. It is evident, however, that the limiting value of p_d is p: whereas the limiting value of $\frac{n}{N_1-n}$ is infinity. Hence depression of vapour pressure and increase of osmotic pressure must diverge more and more rapidly as the concentration increases, although up to moderate concentration they run nearly parallel.

The parallelism of depressions of vapour pressure with osmotic pressures of limited amount has been inferred by the well-known statical thermodynamical method of Arrhenius, or the cyclical thermodynamical method of van 't Hoff. But the mathematical deduction, with the help of these methods, of the vapour pressure equation $\frac{p_1-p}{p_1}=\frac{n}{N}$ is imperfect in the absence of experimental proof that osmotic pressure is proportional to $\frac{n}{N}$; and as a matter of fact this proportionality, in whatever way n and N are defined, does not really exist, as already shown.

The approximate parallelism between depression of vapour pressure and osmotic pressure when the molecular concentration of solute is small as compared with that of solvent is shown by the following table constructed from the data of Berkeley, Hartley, and Burton [1909] for solutions of calcium ferrocyanide. It will be seen, however, that osmotic pressure increases more rapidly, in proportion, than depression of vapour pressure.

TABLE IV. *Depression of Vapour Pressure and Osmotic Pressure of Calcium Ferrocyanide Solutions at* 0°.

Grammes anhydrous calcium ferrocyanide per 100 g. water	Osmotic pressure in atmospheres	Percentage depression of vapour pressure of water	Relative values: Osmotic pressure	Relative values: Depression of vapour pressure
31·388	41·22	3·210	1·000	1·000
39·503	70·84	5·400	1·719	1·682
42·889	87·09	6·557	2·113	2·042
47·219	112·84	8·397	2·737	2·620
49·966	130·66	9·674	3·170	3·014

The case of calcium ferrocyanide is of special interest, since not only is the molecule heavily hydrated ($Ca_2Fe(CN)_2 + 11H_2O$), but, as pointed out in a further paper by Berkeley, Hartley and Stephenson [1909], it is associated in solution, besides being ionised to an extent varying with the concentration. In spite, therefore, of the complexity of the conditions, the expected relation between vapour pressure and osmotic pressure holds good generally, as will be shown in greater detail below (Table V).

When both solvent and solute have a sensible vapour pressure that of the solvent will (within wide limits of concentration) diminish, while that of the solute increases, as $\frac{n}{N_1}$. An interesting special case of vapour pressure is presented by solutions of gases. Here vapour pressure is represented by the partial pressure of the gas in equilibrium with its solution. Most of the common gases are relatively very insoluble in water and other liquids, the attraction of solvent for solute molecules being very small. Hence the value of n is very small in relation to N, so that N, N_1, and $N_1 - n$ are sensibly equal, and it does not matter whether the variation of vapour pressure (or gas pressure) is taken as proportional to $\frac{n}{N_1}$, $\frac{n}{N}$, or $\frac{n}{N_1 - n}$. Henry's law, when put into the older form that the mass of a gas which goes into solution in a given mass of solvent varies directly as the partial pressure of the gas, implies that the vapour pressure of the solute varies as $\frac{n}{N_1 - n}$. The law holds better when stated in the more modern form that concentration of the dissolved gas varies proportionally to concentration of the free gas. This implies that the vapour pressure of the solute varies as $\frac{n}{N}$; and the value of $\frac{n}{N}$ is commonly nearer than that of $\frac{n}{N_1 - n}$ to $\frac{n}{N_1}$. The principles just laid down indicate, however, that concentration of the free gas varies as $\frac{n}{N_1}$, *i.e.* as the ratio of molecular concentration of dissolved gas to total molecular concentration of the solution. This appears, therefore, to be the correct form of Henry's law.

Another interesting special case is that of a vapour in contact with its own pure liquid. Here N_1 does not vary, however much of the vapour condenses. Hence the vapour pressure is constant if the temperature does not vary.

When the solvent liquid contains some other solute which does not enter into chemical combination with the gas, the solubility of the latter will be altered in accordance with Raoult's fundamental law of the relation of vapour pressure to molecular concentration of solutions. Hence most gases are

relatively insoluble in strong aqueous solutions of other solutes and particularly those which, like caustic soda or potash, or calcium chloride, are both very soluble and form hydrates. Such cases as that of sulphuric acid are of special interest in this connection. The commoner gases are about equally soluble in pure water and pure sulphuric acid, but relatively insoluble in solutions of the one liquid in the other, as Bohr found. This is clear evidence of the presence of hydrates in the solutions, with a consequent great diminution of molecular concentration, and therefore of solubility of a gas at a given pressure, since the value of $\frac{n}{N_1}$ is increased.

An important physiological application of Raoult's law is in connection with the solubility of gases in liquids, where there is no solute entering into chemical combination with the gas. Gases, where this condition is fulfilled, are considerably less soluble in blood and other physiological liquids than in pure water, since the molecular concentration, especially with large protein molecules, is considerably less than in water. Owing to certain ill-defined but very adhesive ideas which have become associated with "colloid" chemistry this matter has been much obscured in recent years.

Other inferences, of fundamental importance both in physiology and physical chemistry, are as follows. The diffusion pressure of a substance from its solution into the pure solvent is, in accordance with the reasoning in the previous section of this paper, equal to $\frac{n}{N} P (1 - v)$, where n is moles of the substance per litre, N is moles of the pure solvent; and $1-v$ the proportion of free space in the pure solvent. When the diffusion pressure of the substance is balanced by adding to the pure solvent, or to a solution in it, the requisite number (n_2) of moles of the substance, the diffusion pressure of the substance from the original solution is altered to

$$\frac{n}{N} P \times \frac{N_2}{N} (1 - v),$$

where N_2 is total moles per litre in the new solution. The diffusion pressure of the new solution into the old is $\frac{n_2}{N} P \times \frac{N_1}{N} (1 - v)$, where N_1 is total moles per litre in the old solution. These two values are equal. Hence nN_2 must be equal to n_2N_1, and therefore $\frac{n_2}{N_2}$ must be equal to $\frac{n}{N_1}$. Hence when the ratio of moles of solute to total moles per litre is equal in two solutions in the same solvent they are in diffusion equilibrium for the solute to which the value of n applies, $\frac{n}{N_1} P (1 - v)$, or $\frac{n}{N_1} \cdot 082NT$ may be designated as the diffusion pressure of the substance. But the further deduction has already been made that the vapour pressure (at not too high a pressure) of

a solute varies as $\frac{n}{N_1}$. Hence the vapour pressure of a solute varies as its diffusion pressure when in solution.

Paul Bert was the first to show experimentally that, as has been abundantly confirmed by my own investigations, the physiological action of a gas depends (other things being equal) on its partial pressure. This implies, as already shown, that the action is dependent on the value of $\frac{n}{N_1}$ (n being the molecular concentration of the gas in free solution) in the blood and tissues, and is therefore dependent on the diffusion pressure of the dissolved gas. We have only to substitute "dissolved substance" for "dissolved gas," in order to give Paul Bert's law a far wider scope. It is evidently the variations in *diffusion pressure* of substances within the living organism which matter in a quantitative sense, and to which organic regulation responds. The delicacy with which the partial pressure of CO_2 in the alveolar air, and consequently the diffusion pressure of H_2CO_3 and its ions in the blood, is regulated was shown in 1905 by Priestley and myself in a well-known paper; and in 1912 definite experimental evidence was brought forward by Hasselbalch that it is hydrogen ion concentration to which the respiratory centre responds. It was further shown that there is an almost incredibly delicate regulation of the hydrogen ion concentration (more correctly hydrogen ion diffusion pressure) of the blood [Campbell, Douglas, Haldane and Hobson, 1913].

If we take n as signifying the molecular concentration of free water in an aqueous solution the diffusion pressure of water varies as $\frac{n}{N_1}$, just as does the diffusion pressure of any other substance of which n denotes the molecular concentration. We can then introduce into physiology and physical chemistry the conception of diffusion pressure of water. This is a clear quantitative conception based on the kinetic theory of fluids. The constancy of the depression of freezing-point for blood-serum is well known, and there must of course be a corresponding constancy for osmotic pressure. But depression of freezing-point and osmotic pressure are proportional to $\frac{n}{N_1 - n}$, and not to $\frac{n}{N_1}$, although for low molecular concentrations of a solute the two values are nearly the same. To speak of "the osmotic pressure" of blood and other physiological liquids is clearly, in the light of the conclusions reached above, meaningless except where mechanical pressure is concerned. Experiments recently published by Priestley and myself [1916] on the regulation of excretion of water by the kidneys indicate that diffusion pressure of water in the blood is regulated by the kidneys in a manner comparable as regards extreme delicacy with the regulation of hydrogen ion diffusion pressure.

To demonstrate the existence of blood changes to which the kidneys were re-acting during very rapid excretion of water Priestley had to employ the very delicate electrical conductivity method. This method gave, of course, no direct measure of the diffusion pressure of water in the blood, but in the absence of a sufficiently delicate direct method was very useful for indicating the existence of small changes in the concentration of ionised substances, and thus indirectly of the other substances, which affect the diffusion pressure of water. In this connection it should be noted that for a solution such as blood-plasma or tissue-fluid the value of $\frac{n}{N_1}$ is very different from that of $\frac{n}{N}$, and that for this reason the diffusion pressures of water and other substances are not practically proportional to their concentrations. The molecular concentration (n) of a given substance may differ considerably in different samples of blood-plasma and other physiological fluids, although the value of $\frac{n}{N_1}$ is the same.

4. *Boiling Points of Solutions.*

The connection between the boiling point of a solution and the molecular concentrations of solute and solution follows from the fact that when n gramme-molecules per litre of a solute without sensible vapour pressure are present, the reduction in diffusion pressure outwards of the solvent vapour is $\frac{n}{N} p\,(1 - v)$. To bring the solution to the boiling point the vapour pressure of the solvent must therefore be raised by the amount (p_r) required to neutralise the deficiency, and so raise the vapour pressure to the boiling pressure p. Hence $\left(\frac{N_1 - n}{N}\right)(p + p_r)\,(1 - v)$ must equal $\frac{N_1}{N} p\,(1 - v)$. It follows that p_r must equal $\frac{n}{N_1 - n} p$.

Now although in liquids at the same temperature the total free or externally disposable energy is on an average the same per molecule at each temperature, and varies directly as the absolute temperature, yet the distribution of this energy among the individual molecules is uneven; and in liquids with an appreciable vapour pressure, *i.e.* with part of their molecules in the potentially gaseous state, the potentially gaseous molecules possess more kinetic energy than the liquid molecules. With a given rise of temperature, which of course increases the proportion of potentially gaseous molecules in the liquid, the rise in vapour pressure within the liquid is out of all proportion to the rise in mean intermolecular pressure, or to absolute temperature. If, however, we know the proportional excess of energy required to convert a gramme-molecule of the particular solvent into gas at the temperature

concerned we can calculate the elevation of temperature for a given increase of vapour pressure, as van 't Hoff showed. The expression he deduced for the elevation of temperature giving an increase in pressure of $\frac{n}{N}p$ is $\frac{\cdot 002T^2}{H}$, where H = heat of vaporisation per gramme of solvent at its boiling-point. Thus the elevation of boiling point required to give an increase of $\frac{n}{N_1 - n}p$ is

$$\frac{n}{N_1 - n} N \times \frac{\cdot 002T^2}{H}.$$

This corresponds with the experimental results, and the variable factor is not merely n, as in the van 't Hoff expression, but $\frac{n}{N_1 - n}N$, corresponding to Morse's "weight-normal" concentrations. At great dilutions, however, it becomes, of course, practically indifferent which expression is used.

5. *Melting Points.*

When a crystal at its melting point is in equilibrium with the pure solvent from which it has crystallised we must assume that though molecules are constantly flying out from the crystal in consequence of the continuous molecular agitation due to heat, the number of molecules flying out is balanced by the number flying in from the liquid. Of the molecules which fly out we can further distinguish those which penetrate a given small depth of surrounding liquid from those which are stopped and turned back by collisions with other molecules. The former will be proportional to $1 - v$, in accordance with the reasoning already given; and if p denote the total pressure produced by the flying out of the molecules, the outward diffusion pressure produced by those which penetrate the liquid layer will vary as $p(1 - v)$. This will be balanced by a corresponding pressure in the opposite direction. The diffusion pressure will, in fact, vary just in the same way as if the layer of liquid were a completely permeable membrane.

Now let us suppose that without altering the temperature a solute is introduced into the liquid, and that n, N, N_1 denote gramme-molecules per litre of solute, pure solvent, and solution. The diffusion pressure outwards through the liquid layer will, in accordance with the reasoning in the case of osmotic pressure, alter to $\frac{N_1}{N}p(1 - v)$, and that inwards to $\frac{N_1 - n}{N}p(1 - v)$. Hence there will be an excess outwards of $\frac{n}{N}p(1 - v)$, just as in the case of a semi-permeable membrane. The crystal will therefore melt away.

To make the crystal stable again, we must diminish the free energy of its molecules by lowering the temperature, just as in order to neutralise osmotic pressure we must raise the intermolecular pressure of the solution so as to

neutralise diffusion outwards. The lowering of temperature will, also, just as in the case of osmotic pressure, be proportional to $\frac{n}{N_1 - n}$. The actual lowering of temperature will, as van 't Hoff showed, depend on the latent heat of solution of the solvent, and is given by the expression $\frac{n}{N_1 - n} N \times \frac{\cdot 002 T^2}{H}$, where H = latent heat of fusion, and T = melting point of pure solvent. This expression is the correct one, and differs from van 't Hoff's through the substitution of $\frac{n}{N_1 - n} N$ for n. When m, M, and M_1 are the masses of solute, pure solvent, and solution per litre, we can substitute $\frac{nM}{M_1 - m}$ for $\frac{n}{N_1 - n} N$, as shown above in the case of osmotic pressure.

When the solute, and not the solvent, crystallises out, the same reasoning applies, and the same equation with regard to alterations of crystallisation temperature with concentration of solute, H being taken as latent heat of solution at original temperature, n as existing concentration of solvent molecules, and N, $N_1 - n$ as original and existing concentrations of solute molecules. Hence if we know its solubility and latent heat of solution at any one temperature we can predict its solubilities at other temperatures, provided that no chemical interaction occurs among solute and solvent molecules.

6. *Experimental Verification of the Theory.*

If the foregoing reasoning with regard to osmotic pressure, diminution of vapour pressure, raising of boiling point, and depression of melting point is correct, it should be possible to deduce from any one of these values any of the others for the same concentration and temperature. Even when the concentration is so high that the intermolecular pressure of the solution has begun to alter it should still be possible to make this deduction, since the proportional effect on all the values will be the same. So long as the intermolecular pressures of solutions remain the same as those of the pure solvents it should also be possible, if the concentrations and specific gravities of the solutions are known, to decide as to the molecular weights of the solutes: in other words to deduce the "molecular" osmotic pressures and depressions of vapour pressure, boiling-point and melting-point. Unfortunately the accurate data at present available for testing the reasoning experimentally are somewhat limited, since comparatively few complete observations have been made on sufficiently concentrated solutions; but the chief of these data may now be considered.

As the relative depression of vapour pressure $\frac{p_d}{p}$ equals $\frac{n}{N_1}$, $\frac{p}{p_d}$ equals $\frac{N_1}{n}$,

and $\frac{p}{p_d} - 1$ or $\frac{p-p_d}{p_d}$ equals $\frac{N_1}{n} - 1$ or $\frac{N_1 - n}{n}$. Hence $\frac{p_d}{p-p_d}$ equals $\frac{n}{N_1 - n}$. Hence $\frac{p_d}{p-p_d} \times N$, or N multiplied by the ratio of depression of vapour pressure to vapour pressure of the *solution*, equals $\frac{n}{N_1 - n} \times N$, or gramme-molecules of solute per weight in grammes of one litre of solvent at 0°, and therefore varies as the osmotic pressure, depression of melting-point and rise of boiling-point. Thus we can easily deduce any one of these values from the relative depression of vapour pressure. The molecular osmotic pressure, relative depression of vapour pressure, depression of melting point, and rise of boiling point, are given when the value of $\frac{n}{N_1 - n} \times N$ equals 1. Hence with the molecular fall in the vapour pressure ratio the value of $\frac{p_d}{p-p_d} = \frac{1}{N}$, whereas on the van 't Hoff theory the molecular fall would be given when $\frac{p_d}{p} = \frac{1}{N}$.

Table V shows the osmotic pressures calculated in this way from the relative depression of vapour pressure, as compared with the osmotic pressure actually found by Berkeley, Hartley, and Burton in the experiments already referred to and tabulated in Table IV. For these experiments we have not the data for calculating the molecular weights of the calcium ferrocyanide and its ions.

It will be noticed that the agreement between the calculated values in column 2 and those found is very close, except for the last two observations. In connection with the latter a calculation from the experimental data shows that the observed depressions of vapour pressure must have been appreciably too great; as in spite of the precautions taken the final saturating solution must have increased slightly in concentration, and to an amount which would apparently account for the divergence between calculated and found values. Column 4 shows the increasing divergence between observed values and those calculated on the van 't Hoff theory.

TABLE V. *Depression of Vapour Pressure in relation to Osmotic Pressure.*

	Osmotic Pressure		
Grammes anhydrous $Ca_2Fe(CN)_6$ per 100 g. of total water	Calculated on the theory that $\frac{p_d}{p-p_d} = \frac{n}{N_1 - n}$, and that osmotic pressure $= \frac{n}{N_1 - n} \times 082.NT$	Found	Calculated on the theory that $\frac{p_d}{p} = \frac{n}{N}$, and that osmotic pressure $= 082.NT$. (Van 't Hoff's theory)
31·388	41·21	41·22	39·93
39·503	70·92	70·84	67·18
42·889	87·22	87·09	81·57
47·219	113·88	112·84	104·46
49·966	133·00	130·66	120·34

For aqueous sugar solutions we possess, besides the osmotic pressure observations of Berkeley and Hartley, and of Morse and his co-workers, the determinations by Smits of depressions of vapour pressure, and a series of determinations by various workers of depressions of freezing and elevations of boiling points[1]. As has already been shown, the osmotic pressure results indicate that at 0° cane sugar is present as a pentahydrate in solutions up to high concentrations, but that with rise of temperature the degree of hydration diminishes, and that at 80° the sugar ceases to be hydrated. From similar reasoning it appears that dextrose is present as a di-hydrate at 0°. The following tables show the values calculated for molecular concentrations from the concentrations and experimental results, assuming that the sugar is present in the forms just mentioned. The molecular values are those calculated for a value of 1 of $\frac{n}{N_1 - n} \times N$, or for one gramme-molecule of solute per 1000 grammes of water. The molecular osmotic pressures are divided by T.

Table VI is calculated from the observations by Berkeley and Hartley [1916] of osmotic pressures of cane sugar solution at 0°.

TABLE VI. *Osmotic Pressure of Cane Sugar at* 0°.

Grammes anhydrous sucrose per 100 g. of total water	Gramme-molecules sucrose pentahydrate per 1000 g. free water	Observed osmotic pressure (Berkeley and Hartley)	Molecular osmotic pressure: Calculated from data in columns 2 and 3	Molecular osmotic pressure: Calculated from columns 1 and 3 on Morse's principle	Molecular osmotic pressure: Calculated from molecular concentration on Van 't Hoff's principle
3·393	0·1001	2·23	·0817	·0824	·0838
10·18	0·306	6·85	·0820	·0844	·0894
20·525	0·634	14·21	·0820	·0868	·0976
30·61	0·973	21·87	·0824	·0893	·106
33·945	1·089	24·55	·0823	·0909	·109
81·26	3·020	67·74	·0820	·105	·157
111·73	4·621	100·13	·0795	·112	·190
141·11	6·547	134·84	·0755	·120	·230

When the value of $\frac{n}{N_1 - n} \times N$ (or gramme-molecules solute per weight in grammes of one litre of pure solvent) is 1, the value of p_0 $(= \frac{n}{N_1 - n} \times \cdot 082NT)$ is 22·4 when $T = 273$ (0° C.). The molecular osmotic pressure is thus $\frac{22 \cdot 4}{273} = \cdot 082$. It is evident, therefore, from the fourth column of Table VI (with which Table III should be compared) that up to concentrations of about 100 g. anhydrous sucrose per 100 g. of total water the whole of the sugar is present as pentahydrate when the temperature is 0°. At higher

[1] The latter data are collected in Landolt and Börnstein's *Physikalisch-Chemische Tabellen*, 3rd edition.

concentrations there is evidently a diminishing degree of hydration. It is clear also that consistent figures for molecular osmotic pressure cannot be obtained if the sugar is assumed to be anhydrous (Morse's principle), and that if the molecular osmotic pressure is calculated on van 't Hoff's principle there is no approximation to consistency, except at greater dilutions than the table shows.

Table VII is calculated from measurements by different observers of depressions of freezing point, the first seven being quoted by Landolt and Börnstein, while the last four are from observations by Jones and Getman [1904]. This table confirms the conclusions drawn from Table VI. When the value of $\frac{nN}{N_1 - n}$ (molecules solute per 1000 g. water) is 1 the depression of freezing point ought, according to the foregoing reasoning, to be $\frac{\cdot 001985 T^2}{H}$ and as H (latent heat of fusion of ice) is 79·15, and T is 273, the value of $\frac{\cdot 001985 T^2}{H}$ (*i.e.* the molecular depression of freezing point) is 1·870. The mean of the values calculated for sucrose pentahydrate from the first eight observations is 1·872. In the case of the last three observations the calculated molecular depression diminishes, just as does the calculated molecular osmotic pressure at similar very high concentrations. The last two columns, which may be compared with the last two columns of Table VI, require no comment.

TABLE VII. *Depressions of Freezing-point of Cane Sugar Solutions.*

Grammes anhydrous sucrose per 100 g. of total water	Moles sucrose pentahydrate per 1000 g. free water	Observed depression of freezing-point	Molecular depression of freezing-point: Calculated for sucrose pentahydrate from columns 2 and 3	Molecular depression of freezing-point: Calculated for anhydrous sucrose from columns 1 and 3. (Morse's principle)	Molecular depression of freezing-point: Calculated directly from molecular concentration. (Van 't Hoff's principle)
·04825	·001409	·00264°	1·874°	1·87°	1·87°
·3414	·00999	·01856°	1·858°	1·86°	1·86°
·6875	·02014	·0378°	1·877°	1·88°	1·89°
2·231	·0656	·1230°	1·875°	1·89°	1·91°
4·465	·1320	·2450°	1·856°	1·88°	1·93°
29·82	·944	1·768°	1·873°	2·03°	2·34°
56·18	1·923	3·630°	1·888°	2·21°	2·98°
68·90	2·461	4·612°	1·874°	2·29°	3·29°
84·46	3·178	5·800°	1·825°	2·35°	3·62°
101·99	4·077	7·230°	1·77°	2·43°	4·02°
122·14	5·27	9·13°	1·72°	2·54°	4·57°

Table VIII is calculated from the observations of Smits on the depression of vapour pressure of cane sugar solution at 0°. It will be seen that the observed depressions agree closely with those calculated on the assumption that the sugar is present as pentahydrate and that the depression (p_d) equals

$\frac{n}{N_1} p$, where p is the vapour pressure of pure water at 0° (4·620 mm. of mercury). The molecular depression ratio calculated from the equation $\frac{p_d}{p - p_d} = \frac{1}{N}$ is for an aqueous solution $\frac{1}{55·5} = ·01802$. Column 3 shows that the molecular depression ratios as calculated from the experimental results on the assumption that the sugar is present as pentahydrate correspond with this value. If the depression is calculated on the van 't Hoff theory that $\frac{p_d}{p} = \frac{1}{N}$, or, as already shown in Table IV, on the theory that depression of vapour pressure runs parallel to the osmotic pressure, there is no such agreement.

TABLE VIII. *Depressions of Vapour Pressures of Cane Sugar Solutions at* 0°.

Grammes anhydrous cane sugar per 100 g. total water	Moles sucrose pentahydrate per 1000 g. free water	Observed depression of vapour pressure in mm. of mercury (Smits)	Calculated depression assuming that $p_d = \frac{n}{N_1} p$, and that the sugar is present as pentahydrate	Calculated molecular depression ratio, $\frac{p_d N}{p - p_d} \div \frac{nN}{N_1 - n}$
1·711	·0502	·00422*	·00416	·0182
5·896	·175	·01479	·0145	·0183
15·54	·473	·03972	·0391	·0183
34·52	1·104	·09074	·0906	·01806

* In Smits' paper there is an evident misprint (copied in Landolt and Börnstein's *Tabellen*) of 0·00242 for 0·00422.

Table IX, from the observations collected in Landolt and Börnstein's *Tabellen*, gives elevations of boiling point of cane sugar solutions. At temperatures over 80° cane sugar in solution, as pointed out above, is no longer hydrated. Hence the molecular elevation is calculated for anhydrous cane sugar.

TABLE IX. *Elevations of Boiling point of Cane Sugar Solutions.*

Grammes anhydrous sucrose per 100 g. water	Moles sucrose per 1000 g. of water $\left(\frac{nN}{N_1 - n}\right)$	Observed elevation of boiling-point. Degrees C.	Molecular elevation (observed elevation divided by $\frac{nN}{N_1 - n}$). Degrees C.
2·447	·0715	·035	·49
4·316	·1264	·064	·51
7·25	·212	·103	·49
11·02	·322	·164	·51
16·75	·490	·242	·495
21·68	·634	·317	·50
36·15	1·056	·55	·52
65·47	1·93	1·13	·59
103·4	3·02	2·06	·68
175·1	5·12	3·84	·75
276·2	8·07	6·71	·83

Taking the latent heat of evaporation of water at 100° as 539, the molecular elevation should be $\frac{·001985 \times 373^2}{5·39} = ·512°$. If allowance is made for the increased specific heat of water at 100° this figure is reduced to ·506, which

agrees well with the mean of the figures calculated from the observations up to concentrations of 36 g. per 100 g. of water. At higher concentrations, however, the agreement ceases, presumably because the intermolecular pressure of the solution begins to increase.

Table X is for dextrose solutions, and is calculated from Berkeley and Hartley's determinations of osmotic pressure. They give the concentrations in grammes per litre. The concentrations in molecules per 1000 g. of water are calculated from the specific gravity determinations of Tollens, as given by Landolt and Börnstein. It is evident from the table that at 0° the dextrose is present as a di-hydrate, although, just as in the case of cane sugar, there appears to be some dissociation of the di-hydrate in the strongest solution observed.

TABLE X. *Osmotic Pressures of Dextrose Solutions at* 0°.

Grammes dextrose per litre of solution	Moles dextrose per litre of solution	Molesanhydrous dextrose per 1000 g. of total water	Moles dextrose di-hydrate per 1000 g. free water	Observed osmotic pressure in atmospheres	Calculated molecular osmotic pressure: For dextrose di-hydrate (columns 4 and 5)	Calculated molecular osmotic pressure: For anhydrous dextrose (columns 3 and 5). Morse's principle	Calculated molecular osmotic pressure: From molecular concentration (columns 2 and 5). Van 't Hoff's principle
99·8	0·554	0·591	0·604	13·21	·080	·082	·087
199·5	1·107	1·265	1·327	29·17	·081	·084	·096
319·2	1·772	2·215	2·407	53·19	·081	·088	·110
448·6	2·491	3·454	3·948	87·87	·082	·090	·129
548·6	3·046	4·669	5·612	121·18	·079	·096	·146

The foregoing tables show close agreement between observed values and those calculated on the new principles deduced in the present paper. The agreement affords strong support to these principles, with the corollary that the kinetic theory of fluids can be applied in a simple and direct manner, not merely to very dilute solutions, but also to strong solutions.

CONCLUSIONS.

Boyle's, Charles's and Avogadro's laws can be more correctly stated, and at the same time extended to liquids, in the form: *the intermolecular volume of a given volume of gas or liquid varies inversely as its mean intermolecular pressure, and directly as the absolute temperature, the concentration of gramme molecules, and a constant which is the same for all gases and liquids.*

This generalisation is an extension of the kinetic theory of gases, and is embodied in the equation $P(V - v) = \frac{22{\cdot}4nT}{273}$, or $P(V - v) = {\cdot}082nT$: where P = mean intermolecular pressure in atmospheres: V = volume in litres: v = volume virtually occupied by the molecules themselves, so that $V - v$ = virtual intermolecular volume: n = relative number of molecules

(gramme-molecules); and 22·4 = value of $P\ (V - v)$ when P and $n = 1$ and $T = 273$ (0° C.).

With the help of this equation new quantitative insight is obtained into diffusion pressure, the osmotic pressures, vapour pressures, boiling points, and melting points of solutions, and into other phenomena connected with gases and liquids. It can be shown that:

(1) Diffusion pressure of any one substance between one liquid or gas and another is proportional to the values of its partial pressure p in the one liquid or gas, and $1 - v$ in the other.

(2) The intermolecular pressure is the same for all solutions in the same solvent, up to high concentrations and with wide variations of temperature.

(3) Osmotic pressure is simply the increased intermolecular pressure required to neutralise the excess of diffusion pressure of a pure solvent inwards over that of a diluted solvent outwards, through a membrane permeable to the solvent but not to the solute which dilutes it. This excess is given, up to high concentrations, by the equation $p_0 = \frac{n}{N_1 - n} \cdot 082NT$, where p_0 = osmotic pressure, and n, N, N_1 = gramme-molecules of solute, pure solvent, and solution per litre.

(4) Diminution of vapour-pressure of the solvent and rise of vapour pressure of the solute are proportional to $\frac{n}{N_1}$.

(5) Elevation of boiling point and depression of melting point of the solvent are proportional to $\frac{n}{N_1 - n}$, and can be calculated respectively if the latent heats of boiling or melting of solvent are known.

(6) When the ratio $\frac{n}{N_1}$ is the same in different solutions with the same solvent the substances of which n denotes gramme-molecules per litre (including the solvent) are in diffusion equilibrium, and their mutual diffusion pressure is $\frac{n}{N_1} \cdot 082NT$. This diffusion pressure is of fundamental importance in physiology and physical chemistry, and has often, hitherto, been confused with osmotic pressure.

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XXXVI. STUDIES IN THE ANTITRYPTIC ACTION OF BLOOD SERUM.

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Since Hahn [1897] first definitely observed that the blood serum of normal animals produced an inhibitory action upon the digestive power of trypsin, many investigations have been made into this property, both as regards the nature of the action, and of the particular constituents of the serum which cause it, and as the result several theories have been advanced.

One represents this action as due to the presence in the serum of a true antibody in Ehrlich's sense, analogous to an antitoxin. In support of this numerous statements have been made by Achalme [1901], Jochmann and Kantorowicz [1908], Meyer [1909, 1, 2] and others, that the antitryptic power of the serum of an animal is increased as the consequence of the injection of trypsin into the animal. Meyer [1911] even goes so far as to attribute the presence of antitrypsin in serum to the proteoclastic ferments in the tissue cells acting as antigens, a view which has been reasserted more recently by Stawraky [1914]. The literature however contains much contradictory evidence upon this point, many workers having failed to confirm this increase in antitryptic power after injecting trypsin [Landsteiner, 1907; Doblin, 1909; Rosenthal, 1910, etc.]. Another explanation was given by Rosenthal [1910], who considered that the inhibition could be entirely accounted for by the presence of the products of proteoclastic action. This conclusion, however, was based upon experiments which were not very convincing, and may be dismissed as extremely unlikely; for although the products of a trypsin digest do hinder the action of the enzyme [Bayliss, 1904], yet it is only to a slight extent [Walters, 1912], whilst a very small quantity of serum is capable of producing a very marked inhibition, far too

large to be accounted for in this way. Moreover, the antitryptic power of serum is not removed by dialysis, as would be expected if it were due to amino-acids and other products of protein hydrolysis [Meyer, 1909, 1, 2; Cathcart, 1904; Stawraky, 1914].

The view which has been most generally accepted was put forward by [Hedin 1905; 1906, 1, 2; 1907], who attributed the antitryptic action to the adsorption of the enzyme by some constituent of the serum, and pointed out the similarity of the action of serum upon trypsin to the inhibition of tryptic digestion produced by the addition of animal charcoal.

With regard to the nature of the antitryptic substance in the serum, it was shown by Cathcart [1904] that this property was associated with the albumin fraction of the serum, since on fractionating the serum with ammonium sulphate the antitryptic power was retained by the albumin fraction alone, the other serum proteins being quite inactive. Later investigators have attributed the action to lipoids [Schwarz, 1909] on account of the loss of this power when serum is extracted with chloroform. Jobling and Peterson [1914] consider that it is due to the presence of unsaturated fatty acids which may be extracted from the serum by ether or chloroform, by which means they obtained an extract which possessed an actively antitryptic action. They also found that soaps of unsaturated fatty acids obtained from various sources possessed the property of inhibiting antitryptic digestion.

The antitryptic power of serum has been stated to be increased in certain diseases [Brieger and Trebing, 1908; von Bergmann and Meyer, 1908; Hort, 1909; Golla, 1909, etc.], notably in cancer, in certain acute infections such as pneumonia and typhoid fever, in certain chronic infections such as tuberculosis and syphilis, and in severe anaemias, and suggestions have been made to employ this power as a clinical test. This possibility, with the additional hope, no doubt, of a clue to the cancer problem, has inspired a large amount of research in this direction, and methods have been devised by which the antitryptic power of different sera may be compared quantitatively, the comparison with a normal serum being termed the antitryptic index of the serum.

The methods generally used for obtaining this index are the Loeffler plate method, and the Fuld-Gross method. In the former an increasing series of drops of serum are added to a drop of trypsin placed on a surface of coagulated protein, the whole is incubated and the antitryptic index determined from the relative number of drops required just to prevent the trypsin from digesting away and pitting the underlying protein surface. It is obvious

that this method is very inaccurate since it is almost impossible to judge whether the surface is indented or not.

In the Fuld-Gross method a definite quantity of caseinogen is employed as substrate, and to this is added such an amount of trypsin as will in a given time digest it to such an extent that the addition of a mixture of alcohol and acetic acid just fails to produce a precipitate. To this mixture of caseinogen and trypsin, a definite amount of serum is added and the antitryptic index is obtained by finding how much more trypsin has to be added to digest the caseinogen to the same degree as before in a given time. This method is not capable of any great accuracy since it depends upon an end point reaction, so that when the quantity of substrate (caseinogen) falls below a certain level the rate of digestion is diminished.

To overcome this Golla [1909] employed a method in which equal ferment strengths were allowed to act on a substrate (caseinogen) to which equal units of normal serum and the serum under examination had been added, and continuous observations were made of the amount of caseinogen digested, either by measuring the increase in electrical conductivity, or the loss in viscosity of the mixtures. This method, however, can only give approximately quantitative results when the two sera under comparison have almost the same action, since Hedin [1906, 1] has shown that small quantities of serum produce a relatively larger effect than large quantities, and therefore when two widely different sera are compared the result will depend upon the quantity of the sera employed for the comparison.

In the course of some experiments upon the antitryptic action of serum it became necessary to compare the action of various sera on trypsin and the unsatisfactory methods usually employed made it desirable to reinvestigate the question of the comparison of two sera using more accurate methods of measuring the antitryptic action than those generally employed.

Comparison of the antitryptic action of sera.

Methods employed.

In these experiments equal quantities of a solution of caseinogen (3 per cent. in 0·2 per cent. sodium carbonate) were warmed in a water thermostat at 37° until the temperature of the bath was attained, and the trypsin and serum were then added in the required quantities. After a definite time the quantity of caseinogen digested was estimated by the method used by Hedin [1905] in which a known volume of tannic acid solution was added, the mixture allowed to stand for twelve hours, and the nitrogen determined in a

known volume of the filtrate by the Kjeldahl method. The difference between the nitrogen which escapes precipitation with tannic acid before and after the incubation gives a measure of the digestion. As all results are comparative the numbers given in the experiments detailed below represent cc. of $N/10$ acid corresponding to the ammonia distilled off in the Kjeldahl. All the digestions were carried out in the presence of 0·5 cc. of toluene. In each case care was taken to have such an excess of caseinogen present that the action was directly proportional to the amount of active enzyme, whilst the digestions were only extended over short intervals of time, so that the caseinogen available should not fall sufficiently low to affect the rate of hydrolysis. The enzyme solutions employed were a commercial sample made by Fairchild Brothers and Forster, and preparations made by digesting minced ox or sheep pancreas at 37° in the presence of toluene and chloroform for two or three days, filtering, further digesting the filtrate, and dialysing away the products of digestion against running water. These solutions gave only a faint cloudiness with tannic acid, and only a very faint biuret reaction.

Effects of varying the amount of serum.

It was stated by Hedin [1906, 1, 2] that if trypsin and serum were mixed before being added to the substrate, the neutralising effect of the antibody was larger than when they were added separately; moreover the longer the mixture was kept before being added to the substrate, the greater, up to a certain point, was the amount of trypsin neutralised. Thus the adsorption of the enzyme required a certain time to attain its maximum. From this it seemed probable that if trypsin and serum were added together to caseinogen, the rate of digestion would gradually decrease. The following experiment was done to see if such were the case, goat serum being employed.

The mixtures *A*, *B*, and *C* were incubated at 37°, and 50 cc. of each removed at the intervals stated, 30 cc. tannic acid added and the nitrogen estimated in 60 cc. of the filtrate; the increase in nitrogen is given in cc. 0·1 *N* acid.

A. 250 cc. caseinogen, 7·0 cc. water, 2 cc. trypsin (ox pancreas).
B. 250 „ 6·5 „ 2 „ 0·5 cc. serum
C. 250 „ 6·0 „ 2 „ 1·0 „

Time hours	Total			Rate per hour during preceding interval		
	A	B	C	A	B	C
1	3·4	2·2	1·6	3·4	2·2	1·6
2	6·4	4·2	2·7	3·0	2·0	1·1
4	12·8	8·0	4·7	3·2	1·9	1·0
8	—	15·6	9·0	—	1·9	1·1

The digestion of the caseinogen proceeded in each case at a rate constant within the error of experiment excepting perhaps for a slight irregularity in the first hour after mixing the solutions.

Under these conditions, therefore, a definite rate of reaction may be obtained.

Hedin found that small quantities of serum had a relatively larger inhibitory effect upon the action of trypsin than large quantities; moreover he observed that no amount of serum was able completely to prevent the action of the trypsin. In the experiments quoted however only a few different concentrations of serum were employed. The following experiments were carried out with a large number of concentrations of two sera of different origin to observe the course of the inhibition in each case, and to ascertain whether a definite quantitative comparison of the two sera could be obtained.

Sheep and dog serum were employed.

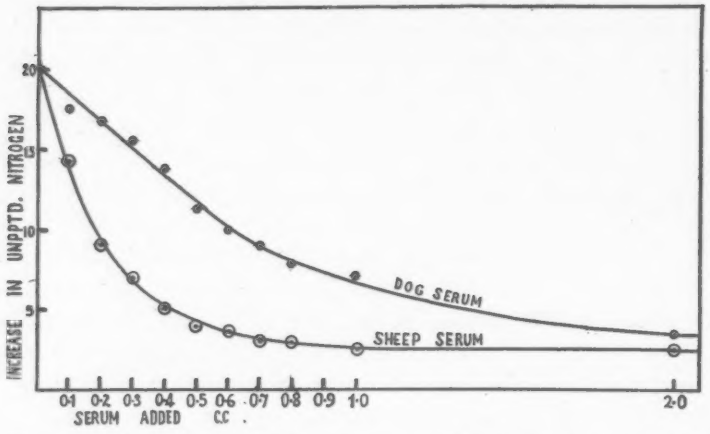
Each mixture consisted of 50 cc. caseinogen solution previously warmed to 37°, 2 cc. trypsin and N cc. serum, the total volume being made up to 55 cc. with water. After four hours 30 cc. of tannic acid were added and the nitrogen determined in 70 cc. of the filtrate. The results are given in the following table.

	Serum added in cc.	Soluble nitrogen produced	
		Sheep serum	Dog serum
1	0	20·4	20·4
2	0·1	14·5	17·8
3	0·2	9·1	17·1
4	0·3	7·0	15·6
5	0·4	5·2	13·8
6	0·5	4·1	11·5
7	0·6	3·7	10·0
8	0·7	3·3	9·0
9	0·8	3·2	8·0
10	1·0	2·6	6·1
11	2·0	2·6	3·7
12	3·0	2·7	3·9

1 cc. trypsin in the same volume in absence of serum gave 10·1 cc., showing that the rate in the absence of serum was proportional to the quantity of trypsin present, and that therefore an excess of caseinogen was present in every case.

From these results it is seen that with increasing amounts of serum the relative inhibition produced by each successive addition gradually falls off, until a point is reached beyond which further additions produce no effect and the curve runs parallel to the base line. Complete inhibition cannot be produced.

The figures are shown graphically on the accompanying diagram.



They show further that the sheep serum produced a much greater inhibitory effect than did the dog serum, both in the early stages and in the final rate. It is apparent, however, that a quantitative comparison of the actions of the two sera cannot be obtained by adding a definite amount of serum to a trypsin digest and comparing the action, since the comparison depends upon which part of the curves representing the reaction are used.

A similar experiment was done with another sample of trypsin to try the effect of very small quantities of serum. The sheep serum was diluted ten times with water, and the dog serum with an equal amount of water.

In each case 2 cc. trypsin were employed and 50 cc. caseinogen, the total volume being 55 cc. The mixtures were incubated for four hours at 37°. The figures in the first column give the volumes of undiluted serum contained in the mixtures.

Serum added cc.	Soluble nitrogen produced	
	Sheep serum	Dog serum
0	11·8	11·8
0·02	12·2	—
0·04	11·6	12·5
0·05	—	12·5
0·06	10·3	—
0·08	9·5	—
0·10	8·6	12·3
0·15	6·5	—
0·20	4·9	11·8
0·25	3·6	—
0·4	—	9·2
1·0	—	4·0

1 cc. trypsin in absence of serum gave 5·5 cc.

The experiment shows that with very small quantities of serum very irregular results were obtained. In fact no definite inhibition was observed with the sheep serum until 0·06 cc. had been added, whilst with the dog serum small quantities produced a slight rise. This may be due to the reaction of the serum since a similar slight rise was produced by adding a very dilute mixture of phosphates so that the mixture when diluted to the same volume as the digest gave the same tint with a suitable indicator, as did 0·05 cc. of serum diluted to the same volume.

The action of the same sample of serum upon two different trypsin solutions of the same digestive power.

During the experiments certain discordant results seemed to point to the fact that the different solutions of trypsin were not always inhibited to the same extent by a single specimen of serum, even when the two solutions of trypsin in the absence of serum were capable of digesting the same quantity of caseinogen in the same interval of time. Experiments were carried out therefore upon this point.

I. One sample of trypsin (1) was prepared from ox pancreas, whilst the other (2) was a commercial preparation of Fairchild's trypsin.

The required volumes of the two trypsin solutions which digested the same amount of caseinogen in the same time were determined in preliminary experiments, which also showed that the quantity of caseinogen was such as not to influence the rate of reaction. In each case 50 cc. of caseinogen were employed, the total volume being made to 60 cc. Sheep serum was used and the mixtures incubated at 37° for five hours.

	Digestion with	
Serum added cc.	Trypsin 1 1·45 cc.	Trypsin 2 1 cc.
0	20·0	19·9
0·1	14·1	10·0
0·2	10·3	7·0
0·5	5·0	4·3

II. A similar experiment was carried out with two different samples of trypsin from ox-pancreas and sheep serum—five hours at 37°.

	Digestion with	
Serum added cc.	Trypsin A 2 cc.	Trypsin B 1·6 cc.
0	17·6	17·7
0·1	13·1	11·3
0·2	11·0	7·1
0·5	5·9	4·0

III. A similar experiment with goat serum and two specimens of trypsin (ox pancreas)—three hours at 37°.

Serum added cc.	Trypsin C 4 cc.	Trypsin D 1 cc.
0	12·5	12·6
0·2	8·2	7·6
0·4	5·7	5·7
0·6	4·6	5·0
2·0	3·6	3·5

IV. A similar experiment with goat serum and two more trypsin preparations from ox pancreas—three hours at 37°.

Serum added cc.	Trypsin E 0·7 cc.	Trypsin F 2·6 cc.
0	16·0	16·5
0·2	12·2	12·0
0·4	9·8	10·3
0·6	7·4	8·9
1·0	4·8	7·3
4·0	4·8	7·3

In experiments I and II the two trypsin solutions in the absence of serum digested the same quantity of caseinogen, yet the effect of adding varying quantities of the serum to each was very different. On the other hand in experiment III the effect on the two preparations was practically the same. In experiment IV, again, a considerable difference was noticed as the quantity of serum was increased.

These experiments show, therefore, that equal quantities of a serum may not always produce the same inhibitory effect upon different preparations of trypsin although the enzyme solutions have the same proteoclastic power as measured by the digestion of caseinogen.

In obtaining the antitryptic index of a serum a sample of enzyme is generally employed of such a strength that it will just digest away a given amount of caseinogen in a given time, as in the Fuld-Gross method. As shown above the effect of serum upon this may vary with the particular sample of enzyme used, and it is obvious therefore that the comparison of the antitryptic action of a different serum made at different times with different trypsin preparations, even though of standard strength, may yield erroneous results, and many of the contradictory results found in the literature may probably arise in this way.

From these experiments the conclusion must be drawn that the antitryptic action of the two sera may be compared only by observing the effect of varying quantities of each upon the same sample of trypsin, and that only a qualitative comparison is then possible.

Is this action due to the presence of zymoid in the enzyme preparations?

The different inhibitory powers of the same serum upon different preparations of trypsin might be explained by the presence in the solution of enzyme so altered that it is incapable of digesting caseinogen but is yet able to enter into association with the inhibitory substance in the serum. Evidence of the existence of a modified enzyme which would still combine with antibody but had lost the power of clotting milk, has been put forward by Korschun [1902] in the case of rennet, whilst Bayliss [1904] obtained facts which suggested the production of a similar modification of trypsin by warming to 25° for a day or two. For these modifications Bayliss suggested the name of zymoid. Furthermore Bearn and Cramer [1907] stated that when trypsin was heated to about 50° or 60° the presence of a zymoid was apparent. Experiments were carried out therefore, to see if trypsin could be altered in this manner by heating.

A solution of the trypsin was heated for two hours at 60°, and thus rendered inactive; the addition of this to a mixture of caseinogen, trypsin and serum was without effect upon the quantity of caseinogen digested. Solutions of trypsin were kept at different temperatures, between 30° and 40° for varying times so as to lessen their activity, and quantities of each of similar digestive power to a given volume of the original trypsin, were tested upon caseinogen in the presence of varying quantities of the same serum. In every case it was found that the power of associating with the serum was lost in proportion to the loss of activity. No evidence therefore could be obtained of the presence of zymoid.

Variations in the antitryptic action of serum.

As already stated the antitryptic action of serum has been proposed for diagnostic purposes in disease, a proposal which assumes that the sera of normal animals of the same species have the same inhibitory action upon trypsin. On the other hand it has been contended that considerable variations do occur in the action of normal serum even in the same animal.

In the following experiment the inhibitory action of the serum from three dogs was compared to see if naturally occurring differences could be found. To avoid errors due to different trypsin preparations, the digestions were carried out at the same time using the same trypsin solution. Parallel series of mixtures were incubated for three hours at 37°, and the extent of digestion of the caseinogen measured as before. Each mixture contained 50 cc. casein-

ogen, 5 cc. trypsin and varying amounts of fresh serum, the total volume being constant. The results are given in the table.

Two of the dogs (1 and 2) were young dogs, about four months of age, and the third was a fully grown female. For comparison also the action of the serum of a goat is given.

Serum added cc.	Soluble nitrogen produced			
	Dog 1	Dog 2	Dog 3	Goat
0	12·6	12·6	12·6	12·6
0·2	10·4	9·6	10·2	8·2
0·4	7·6	7·2	7·6	5·7
1·0	5·1	5·2	5·5	4·0
2·0	4·2	4·2	4·4	3·4
3·0	4·1	4·0	4·2	3·2

The inhibitions produced by the sera from the three dogs were thus substantially the same in every case; the goat serum, however, showed a more marked difference from the others; in an earlier experiment we have already seen that serum from dog and sheep were different in their behaviour.

Attempted immunisation against trypsin.

The question whether the injection of enzyme into an experimental animal is followed by the production of true antibodies has given rise to a considerable amount of work. It has been pointed out by Bayliss [1914, p. 127], that the evidence tends to show that enzymes are not proteins, and as it is very doubtful whether any other substances are capable of acting as antigens and producing antibodies when injected into living organisms, these are *prima facie* grounds for doubting the capacity of enzymes to act as antigens.

The first "anti-enzyme" was described by Hildebrandt [1893], who stated that the injection of emulsin into animals was followed by the appearance in the serum of the power of inhibiting the action of emulsin on glucosides. Bayliss [1912], however, found that this action was due to the diminution of acidity of the mixture, and he showed that when the hydrogen ion concentration was brought back to its original value the effect disappeared, whilst the same inhibitory effect could be produced by making the emulsin to the same hydrogen ion concentration as that produced by adding the immune serum. In the case of the "anti-rennet" produced by immunising animals with rennet, Hedin [1911, 1912] states that it is not a true antibody, whilst Thaysen [1915] says that it may be completely accounted for partly by adsorption of the enzyme, and partly by the effect of change of hydrogen ion concentration.

In the case of trypsin, as stated earlier, many statements have been made to show that an antitrypsin can be produced by the injection of the enzyme, whilst on the other hand there are recorded numerous failures to obtain it. In the case of trypsin the difficulty lies in the presence of the antitryptic property in normal serum. In reviewing the literature it appeared probable that many of the contradictory results might be accounted for by several causes. In many cases the "immune" sera from the experimental animals were tested against the serum of a normal animal of the same species, and it is possible that the antitryptic power of normal animals might vary with the individual. On the other hand, if the blood of the same animal were employed and its antitryptic activity tested before and after injection, the tests would be made usually by the Fuld-Gross method with the fresh serum and thus at different times and possibly with different specimens of trypsin. The latter although of standard activity as regards action on the substrate, might, as has already been shown, be adsorbed to a different degree by the serum, so that it might appear as if the activity of the serum had been altered. In the following experiments attempts have been made to avoid these difficulties by testing the serum of the animal before and after injection against the same trypsin solution. The solutions of trypsin used for injection were rendered as sterile as possible by keeping them for a day or two in the presence of toluene, the latter being then removed by a rapid current of air previously passed through sterile cotton wool. Before the injections were made the experimental animal was bled generally twice with an interval of a week, to obtain two samples of normal serum, which were preserved as described below. The solutions were then injected intravenously, several injections being made at suitable intervals. After a certain time the animal was again bled and the serum collected. In this way two specimens were obtained of the normal serum of the animal for comparison with the serum of the same animal after immunisation, the object being to try their inhibitory effect upon the same sample of trypsin, and to ascertain whether any difference in activity was produced and if so whether this difference was greater than such difference as might exist between the two samples of normal serum. In each experiment the antitryptic action of the serum was tested against the same trypsin preparation as was used for the injection. The sera as they were obtained were dried rapidly by the method recommended by Chapman [1905]. This was done by spreading 10 cc. over the surface of a tared flat glass dish, which was then kept in an evacuated desiccator containing sulphuric acid and placed in an incubator at a temperature of 37°. Within 24 hours the serum dried up. The dish and

contents were then weighed and the dried mass powdered and preserved in a dry atmosphere[1] in the ice chest until required, when the powder was dissolved in water and made up to the volume of the original serum from which it had come. The serum when preserved in this manner retains its activity for a long time. In every case the sera were neutralised to litmus paper by the addition of sodium dihydrogen phosphate, before being tested. The tests were made by adding an increasing series of quantities to mixtures of trypsin and caseinogen, incubating at 37°, precipitating with tannic acid, and estimating the nitrogen in the filtrate. The following experiments with the same samples of serum dried and undried show that the drying had not produced any appreciable effect upon the antitryptic activity. The undried serum was kept on ice during the time taken in the drying of the other portion.

I. Each mixture consisted of 25 cc. caseinogen, 1·2 cc. trypsin (sheep pancreas), and varying amounts of the serum, the total volume being made to 30 cc. with water. After 18 hours at 37°, 15 cc. of tannic acid were added to each and the nitrogen estimated in 20 cc. of the filtrate. In the absence of serum 30·2 cc. were obtained.

Serum added cc.	Digestion with	
	Dried serum	Undried serum
0·5	6·0	6·0
1·0	3·6	4·0
2·0	3·1	3·2

II. A similar experiment with 1·2 cc. trypsin (ox pancreas) and sheep serum. In the absence of serum the digestion corresponded to 24·5 cc.

Serum added cc.	Digestion with	
	Dried serum	Undried serum
0·05	22·7	23·1
0·10	21·7	21·3
0·30	14·1	13·9
0·50	10·1	10·1
1·00	6·1	6·4

III. A goat (I) was injected intravenously at weekly intervals with the following quantities of trypsin (commercial sample of Grübler) dissolved in

[1] On several occasions the serum powder when preserved for some time without special precautions being taken to keep it dry, was found to have become partially insoluble in water. Professor Chapman has informed me that he has noticed the same thing, and he attributes it to the effect of moisture which causes the proteins of the serum to coagulate. This was confirmed by an experiment in which two portions of the same serum powder were preserved, the one in a desiccator containing calcium chloride, and the other in moist air. In the latter case the serum soon became insoluble, whilst in the former it remained perfectly soluble. When precautions were taken to keep the powders dry this insolubility was never observed.

70 cc. of normal saline in each case; 0·4, 0·4, 0·4, 0·6, 1, 1, 1, 1, 1, g. After another week a sample of serum was obtained. Two samples of normal serum were collected before the injections for comparison. The results are given in the following table. Each mixture contained 25 cc. caseinogen, 5 cc. trypsin (1 g. in 50 cc.) and the quantities of serum given below, the total volume being constant.

	Digestion in the presence of serum		
Serum added	Before 1	Before 2	After injection
0·5	17·0	17·4	18·0
1·0	10·1	10·3	10·5

In absence of serum digestion = 28·6.

IV. A goat (II) received five intravenous injections of trypsin solution prepared from ox pancreas, 5, 7·5, 10, 20, 20 cc. being injected at weekly intervals. Two samples of normal and one of "immune" serum were collected.

The action on the enzyme is given below, each mixture containing 2·5 cc. trypsin, 25 cc. caseinogen and varying amounts of the serum. The mixtures were incubated at 37° for 18 hours.

	Digestion in presence of serum		
Serum added cc.	Before 1	Before 2	After injection
0·5	9·1	8·7	9·4
5	2·5	2·4	3·1

In the absence of serum 18·8 cc. were obtained.

V. A goat (III) received three injections intravenously of 20 cc. of trypsin (ox pancreas). Only one sample of normal serum was used. To test the antitryptic activity 2·5 cc. trypsin and 25 cc. caseinogen were digested with the sera, as given below, at 37° for 18 hours.

	Digestion in the presence of serum	
Serum added cc.	Before	After injection
0·01	18·0	18·0
0·05	17·5	17·5
0·10	16·7	16·5
0·25	15·2	14·6
0·50	10·1	9·1
1·00	6·1	5·7

In the absence of serum 18·9 cc. were obtained.

VI. In this experiment a goat was injected intraperitoneally with 20 cc. of trypsin (ox pancreas) on three successive days and was bled after seven days. The injections were not followed by any untoward results in the animal. The mixtures for testing the sera contained 2·5 cc. trypsin, 25 cc. caseinogen and varying amounts of serum, and were kept at 37° for 16 hours.

Serum added cc.	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·01	18·4	18·1	18·2
0·05	16·8	16·4	17·5
0·10	15·9	15·2	16·3
0·25	10·6	11·0	13·0
0·50	6·5	6·2	6·2
1·00	5·0	4·8	4·5
2·50	4·7	4·6	4·1
5·00	3·9	3·1	3·4

In absence of serum 18·9 cc. were obtained.

Intraperitoneal injection had produced no increased antitryptic action.

The following two experiments were made upon sheep with a sample of trypsin prepared from the pancreas of an animal of the same species.

VII. A sheep (I) was injected intravenously at weekly intervals with 40, 70, 75 cc. of trypsin solution (sheep pancreas). The mixtures for testing the sera containing 1·2 cc. trypsin, 25 cc. caseinogen and varying volumes of serum were incubated at 37° for 18 hours.

Serum added cc.	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·5	16·2	17·6	16·0
1·0	3·2	3·3	4·0
2·0	2·9	3·1	3·1

In absence of serum—30·2 cc.

The animal then received two further injections of 75 and 175 cc. trypsin.

Serum added cc.	Digestion	
	Before	After injection
0·1	24·8	24·0
0·5	17·7	18·6
1·0	5·9	5·1
2·0	3·0	3·3

In the absence of serum = 29·0 cc.

VIII. A sheep (II) was injected intravenously with a solution of trypsin from sheep pancreas receiving the following doses at weekly intervals—40, 70, 75, 75 cc.

Serum added cc.	Digestion in presence of serum		
	Before 1	Before 2	After injection
0·1	26·5	26·5	26·0
0·2	20·1	24·0	24·4
0·4	19·6	20·5	21·0
1·0	10·3	10·5	11·6
2·0	5·9	6·1	6·3

In absence of serum = 30 cc.

A further injection of 200 cc. of a fresh preparation from sheep pancreas was then given and the serum collected after 14 days and tested against the original normal serum.

Serum added cc.	Digestion	
	Before 1	After injection
0·1	8·3	9·1
0·25	4·7	4·8
0·50	4·5	4·4
1·00	4·5	4·5
1·50	4·2	4·1

In absence of serum = 23·4 cc.

In all these experiments there was no greater difference observed between the antitryptic action of the serum of the animals before and after the injections, than was found between the two samples of serum from the same animal before injection. There was therefore no evidence of the production of any antitrypsin by the injection of the enzyme.

These experiments were extended by examining the sera of the animals to see if the injections had given rise to the formation of a specific precipitin. The serum of goat (I) gave no precipitate when mixed with the trypsin solution used for the injection; on the other hand, after the injection of the trypsin the serum gave a precipitate with dilutions of the trypsin down to 1 in 1000.

The serum of goat (II) injected with ox pancreas trypsin before injection gave no precipitate with the trypsin solution. After injection, however, it gave a distinct precipitate when mixed with the trypsin solution in dilutions down to 1 in 100.

Similarly with goat (III) intraperitoneal injections with trypsin (ox pancreas) produced a faint precipitin reaction.

The sera of the two sheep before injection were also compared with the sera obtained after the final injection of trypsin. On mixing with the solution of trypsin employed, a distinct precipitate was noticed in the presence of 1/100 and 1/1000 respectively of enzyme solution, whereas with the normal sera the mixture remained quite clear.

The last two cases are of interest as an instance of a precipitin being produced by injecting the extract of the pancreas of an animal of the same species.

From these results it is seen that although there was no increased antitryptic power observed in the serum due to the injection, yet a precipitin was produced. They thus show a similarity to the experiments quoted by Bayliss [1912] with emulsin where a precipitin was also produced when enzyme preparations were injected, although he was able to demonstrate that

no true antibody to the enzyme was produced at the same time, and a similar conclusion must be drawn here as was done by Bayliss that the antigen to this precipitin was most probably some protein accompanying the trypsin and not the enzyme itself. These experiments contain therefore additional evidence that the enzyme trypsin is not a protein.

The results obtained do not bear out the statement of Porter [1914] that trypsin is inhibited by a specific precipitate. In her case the effect was compared of adding egg white to a mixture of trypsin and serum from a rabbit immunised with egg white, with that produced by adding egg white to a mixture of trypsin and normal serum. The differences shown were exceedingly small however, and no attempt was made to ascertain whether they could not be accounted for by changes in alkalinity of the mixture.

Summary.

The observations herein recorded confirm those of Hedin and show that when increasing amounts of blood serum are added to a mixture of trypsin and excess of caseinogen the inhibitory effect on the trypsin, produced by equal increments of serum, decreases as the total quantity of serum present gets larger, until a point is reached beyond which the further addition of serum produces no further decrease in the action of the enzyme. In no case was it found possible completely to inhibit the action of trypsin by the addition of serum.

Different preparations of trypsin of equal digestive power upon caseinogen are not always inhibited to the same extent by the addition of equal quantities of the same serum.

Experiments to ascertain whether this was due to the presence of a zymoid—*i.e.* altered enzyme which had lost its power of hydrolysing caseinogen but was still able to form an association with the antibody in the serum—were unsuccessful.

The experiments show that in making a comparison of the effect of different sera upon trypsin, it is necessary to employ the same individual preparation of trypsin, and even then only a qualitative result is possible. The so-called antitryptic index, therefore, is not a definite constant for a serum.

In the experiments quoted in this paper the sera of normal animals of the same species did not show any marked variations in "antitryptic action," nor was any great change observed in the serum drawn at different times from the same animal; on the other hand, marked differences were observed between the actions of the serum from different animals (goat, sheep, dog).

It is shown that the possible errors in the methods usually employed for

comparing the effect of sera upon trypsin may account for the contradictory results obtained by so many workers upon the question whether the injection of trypsin into experimental animals is followed by an increase in the antitryptic action of the serum.

Experiments are recorded in which attempts were made to increase the antitryptic action of the serum of an animal by injecting the enzyme intravenously or intraperitoneally, precautions being taken in testing the serum to avoid the errors suggested by the previous experiments.

The action of the "immune" serum in each case was compared with that of two samples of serum drawn from the same animal before the injections were made, and the comparison was made in parallel series of increasing amounts of serum, the same solution of trypsin being employed and the experiments being carried out at the same time. In no case was any increased action observed to follow the injections.

The injections, however, caused the production of a precipitin, which must therefore be attributed to the presence of protein accompanying the enzyme and not to the enzyme itself. These experiments, therefore, bring forward some evidence that trypsin is not a protein.

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